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<b>(21) International Application Number:</b> PCT/US99/00781 <b>(22) International Filing Date:</b> 14 January 1999 (14.01.99)  <b>(30) Priority Data:</b> 09/007,064 14 January 1998 (14.01.98) US  <b>(71)(72) Applicant and Inventor:</b> LAKOWICZ, Joseph, R. [US/US]; 10037 Fox Den Road, Ellicott City, MD 21042 (US).  <b>(74) Agents:</b> REPPER, George, R. et al.; Rothwell, Figg, Ernst & Kurz, Columbia Square East, Suite 701, 555 13th Street, N.W., Washington, DC 20004 (US).		<b>(81) Designated States:</b> CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>With amended claims.</i>
<b>(54) Title:</b> METHOD AND COMPOSITION FOR DETECTING THE PRESENCE OF A NUCLEIC ACID SEQUENCE IN A SAMPLE		
<b>(57) Abstract</b> <p>A method for detecting the presence of a nucleic acid sequence in a sample includes the step of coupling a fluorescent metal-ligand complex to an oligonucleotide having a sequence complementary to the nucleic acid sequence to form a probe. The probe is added to a sample that contains the nucleic acid sequence to form a mixture containing a reaction product. The mixture is exposed to an exciting amount of radiation. The fluorescence of the metal-ligand complex is detected, and the presence of the nucleic acid sequence is determined based on the fluorescence of the metal-ligand complex. A composition for detecting the presence of a nucleic acid sequence, includes a fluorescent metal-ligand complex coupled to an oligonucleotide having a sequence complementary to the nucleic acid sequence. A diagnostic kit for detecting the presence of a nucleic acid sequence, includes a fluorescent metal-ligand complex coupled to an oligonucleotide having a sequence complementary to the nucleic acid sequence.</p>		

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METHOD AND COMPOSITION FOR DETECTING THE  
PRESENCE OF A NUCLEIC ACID SEQUENCE IN A SAMPLE

BACKGROUND OF THE INVENTION

Field of the Invention

5       The present invention is in the field of detecting  
the presence of a nucleic acid sequence in a sample.

Description of the Background Art

10       Fluorescence *in situ* hybridization ("FISH") is widely  
used in medical diagnostics. Present technology for *in*  
*situ* hybridization includes the use of fluorescence probes  
such as fluorescein and rhodamine and detection of  
hybridized DNA by localization of the product of an enzyme  
catalyzed reaction.

15       The following references describe known fluorescence  
*in situ* hybridization techniques:

Swiger, R.R., and Tucker, J.D., "Fluorescence *In Situ*  
Hybridization: A Brief Review," *Environmental and*  
*Molecular Mutagenesis* 27:245-54 (1996).

20       Schrock, E., du Manoir, S., Veldman, T., Schoell, B.,  
Wienberg, J., Ferguson-Smith, M.A., Ning, Y., Dedbetter,  
D.H., Bar-Am, I., Soenksen, D., Garini, Y., and Ried, T., "  
Multicolor Spectral Karyotyping of Human Chromosomes,"  
*Science* 273:494-98 (1996).

25       Lewis, R., "Chromosome Charting Takes a Giant Step,"  
*Photonics Spectra* 48-50 (1996).

Fox, J. L., Hsu, P., Legator, M., Morrison, L.E., and  
Seelig, S.A., "Fluorescence *In Situ* Hybridization:

Powerful Molecular Tool for Cancer Prognosis," *Clinical Chemistry* 41:1554-59 (1995).

Nederlof, P.M., van der Flier, S., Wiegant, J., Raap, A.K., Tanke, H.J., Ploem, J.S., and van der Ploeg, M.,  
5 "Multiple Fluorescence In Situ Hybridization," *Cytometry* 11:126-31 (1990).

Siadat-Pajouh, M., Ayscue, A.H., Periasamy, A., and Herman, B., Introduction of a Fast and Sensitive  
Fluorescent In Situ Hybridization Method for Single-copy  
10 Detection of Human Papillomavirus (HPV) Genome," *The Journal of Histochemistry and Cytochemistry* 42:1503-12 (1994).

Kearns, W.G. and Pearson, P.L., "Fluorescent In Situ Hybridization Using Chromosome-Specific DNA Libraries,"  
15 *Methods in Molecular Biology* 33:15-22 (1994).

Tkachuk, D.C., Pinkel, D., Kuo, W., Weier, H., and Gray, J.W., "Clinical Applications of Fluorescence in situ Hybridization," *GATA* 8(2):67-74 (1991).

Denijn, M., Schuurman, H., Jacobse, K.C., and Weger, R.A., "In Situ hybridization: A valuable tool in  
20 diagnostic pathology," *APMIS* 100:669-681 (1992).

McNeil, J.A., Villnave Johnson, C., Carter, K.C., Singer, R.H., and Bentley Lawrence, J., "In Situ Hybridization," *GATA* 8(2):41-58 (1991).

25 Schwarzachter, T., and Heslop-Harrison, J.S., "Direct Fluorochrome-Labeled DNA Probes for Direct Fluorescent In Situ Hybridization of Chromosomes," *Methods in Molecular*

*Biology, Vol. 28: Protocols for Nucleic Acid Analysis by Nonradioactive Probes* 167-76 (1994).

5 Durrant, J., Brunning, S., Eccleston, L., Chadwick, P., and Cunningham, M., "Fluorescein as a label for non-radioactive *in situ* hybridization," *Histochemical Journal* 27:94-99 (1995).

None of these papers mentions the use of metal-ligand complexes in fluorescence *in situ* hybridization to detect the presence of a nucleic acid.

10 One limitation of prior art DNA hybridization is the low levels of light available from commonly used fluorophores, the presence of significant background fluorescence which limits sensitivity, and photobleaching of the probes. The resulting fluorescence is typically on  
15 a nanosecond time scale, which is also the decay time of the commonly used fluorophores. In addition, the commonly used fluorophores display small Stoke's shifts making it difficult to detect their fluorescence in the presence of a fluorescent background. There is also a need for  
20 fluorophores with greater resistance to fading and increased shelf life, i.e., archivability of the slides.

There is extensive literature regarding the spectral properties of metal-ligand complexes. The following is a list of papers regarding metal-ligand complexes:

25 Maestri, M., Sandrini, D., Balzani, V., Maeder, U. and von Zelewsky, "Absorption Spectra, Electrochemical Behavior, Luminescence Spectra, and Excited-State

Lifetimes of Mixed-ligand Ortho-Metalated Rhodium(III) Complexes," *Inorg. Chem.*, 26:1323-1327 (1987).

Sutin, N. and Creutz, C., "Properties and Reactivities of the Luminescent Excited States of Polypyridine Complexes of Ruthenium(II) and Osmium(II)," *Inorg. & Organometall. Photochem.*, Chap. 1, pp. 1-27 (1978).

Hager, G.D., Watts, R.J. and Crosby, G.A., "Charge-transfer Excited States of Ruthenium(II) Complexes. Relationship of Level Parameters to Molecular Structure," *J. Am. Chem. Soc.*, 97:7037-7042 (1975).

Orellana, G. and Braun, A.M., "Quantum Yields of <sup>3</sup>MLCT Excited State Formation and Triplet-Triplet Absorption Spectra of Ruthenium(II) Tris-Chelate Complexes Containing Five- and Six-Membered Heterocyclic Moieties," *J. Photochem. Photobiol. A. Chem.*, 48:277-289 (1989).

Harrigan, R.W. and Crosby, G.A., "Symmetry Assignments of the Lowest CT Excited States of Ruthenium(II) Complexes Via a Proposed Electronic Coupling Model," *J. Chem. Phys.*, 59(7):3468-3476 (1973).

Yersin, H. and Braun, D., "Isotope-Induced Shifts of Electronic Transitions: Application to [Ru(bpy-h<sub>8</sub>)<sub>3</sub>]<sup>2+</sup> and [Ru(bpy-d<sub>8</sub>)<sub>3</sub>]<sup>2+</sup> in [Zn(bpy-h<sub>8</sub>)<sub>3</sub>] (ClO<sub>4</sub>)<sub>2</sub>," *Chem. Phys. Letts.*, 179(1,2):85-94 (1991).

Coe, B.J., Thompson, D.W., Culbertson, C.T., Schoonover, J.R. and Meyer, T.J., "Synthesis and Photophysical Properties of Mono(2,2',2'-Terpyridine)

Complexes of Ruthenium(II)," *Inorg. Chem.*, 34:3385-3395 (1995).

Lees, A.J., "Luminescence Properties of Organometallic Complexes," *Chem. Rev.*, 87:711-743 (1987).

5 DeArmond, M.K. and Carlin, C.M., "Multiple State Emission and Related Phenomena in Transition Metal Complexes," *Coordination Chem. Rev.*, 36:325-355 (1981).

Kondo, T., Yanagisawa, M. and Fujihira, M., "Single Exponential Decay for the Luminescence Intensity of Ru(bpy)<sub>3</sub><sup>2+</sup> Complex in Langmuir-Blodgett Films," *Chem. Letts.*, 1639-1993 (1993).

None of the above references suggest use of metal-ligand complexes in fluorescence *in situ* hybridization.

15 There remains a need in the art for improved methods of detecting the presence of nucleic acid sequences.

#### SUMMARY OF THE INVENTION

In accordance with the present invention, a method for detecting the presence of a nucleic acid sequence in a sample includes the step of coupling a fluorescent metal-ligand complex to an oligonucleotide having a sequence, complementary to the nucleic acid sequence to form a probe. The probe is added to a sample that contains the nucleic acid sequence to form a mixture containing a reaction product... The mixture is exposed to an exciting amount of radiation. The fluorescence of the metal-ligand complex is detected, and the presence of the nucleic acid

sequence is determined based on the fluorescence of the metal-ligand complex.

Also in accordance with the present invention a composition for detecting the presence of a nucleic acid sequence includes a fluorescent metal-ligand complex coupled to an oligonucleotide having a sequence complementary to the nucleic acid sequence.

Further in accordance with the present invention a diagnostic kit for detecting the presence of a nucleic acid sequence includes a fluorescent metal-ligand complex coupled to an oligonucleotide having a sequence complementary to the nucleic acid sequence.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 schematically shows an instrument based on a simple light source and a CCD detector. This figure shows aspects of the invention based on the spectral properties of the metal-ligand complexes. The simple light source is pulsed at sufficient speed, and the detector is gated to provide high sensitivity imaging with suppression of background fluorescence.

FIG. 2 is a graph depicting background suppression with a long lifetime metal-ligand complex.

FIG. 3 graphically shows exciting metal-ligand complexes with a wide variety of simple light sources.

FIG. 4 graphically shows the high photochemical stability of the metal-ligand complexes. The high photochemical stability increases detection sensitivity



and allows detection of the smallest number of gene copies in a chromosome sample. The high chemical stability of the metal-ligand complexes facilitates archiving pathology samples for future analysis.

5           FIG. 5 graphically shows that the emission maximum of the metal-ligand complex can be altered by selection of the metal. Spectral karyotyping or multiplex FISH allow the identification of all 23 human chromosomes based on the emission spectra of the FISH probes. Metal-ligand  
10 complexes may be used in spectral karyotyping or multiplex FISH by utilizing metals with distinct emission spectra.

          FIG. 6 graphically shows that the decay time of a metal-ligand complex can be altered by selection of the ligand. Lifetime imaging using metal-ligand complexes may  
15 be used to distinguish sites on a chromosome.

          FIG. 7 is a formula representation of a fluorescent nucleotide for use in FISH. A fluorophore can be attached to the base.

          FIG. 8 depicts compounds for labeling DNA. DNA can  
20 be labeled using fluorescent primers (top) or using labeled nucleotide triphosphates. DNA can also be labeled on the 5' end via a thiophosphate linkage.

          FIG. 9 shows metal-ligand complexes which are reactive with sulfhydryl or amino groups and hence can be  
25 coupled to nucleic acids.

          FIG. 10 depicts a representative synthesis of one reactive metal-ligand complex.

FIGS. 11 and 12 describe show spectral properties of other metal-ligand complexes. These figures show that one can obtain high quantum yield metal-ligand complexes for high sensitivity detection.

5        FIG. 13 graphically depicts methods to detect DNA hybridization by energy transfer. "D" is the donor, "A" is the acceptor or quencher, and "I" is the intercalating dye. The dyes can be free dyes, which intercalate as in Figure 13(c), or covalently bound intercalators as in  
10      Figure 13(d).

FIG. 14 depicts metal-ligand complexes containing the dppz ligand which can intercalate into double helical DNA.

FIG. 15 schematically shows the release of donor quenching during polymerase chain reaction.

15        FIG. 16 schematically and graphically shows the energy transfer from a metal- ligand complex donor.

FIG. 17 is the molecular structure for  $[\text{Re}(2,9\text{-dimethyl-4,7-diphenyl-1,10-phenanthroline})(\text{CO})_3(\text{isonicotinic acid})]^+$ .

20        FIG. 18 is the molecular structure for  $[\text{Ru}(4,7\text{-diphenyl-1,10-phenanthroline})_2(4,4'\text{-dicarboxylic acid-2,2'-bipyridine})]^{2+}$ .

FIG. 19 is the molecular structure for  $[\text{Ru}(4,7\text{-diphenyl-1,10-phenanthroline})_2(4\text{-methyl,4'-carboxylic acid-2,2'-bipyridine})]^{2+}$ .  
25

FIG. 20 is the molecular structure for  $[\text{Ru}(4,7\text{-diphenyl-1,10-phenanthroline}(\text{SO}_3\text{Na})_2)_2(4,4'\text{-dicarboxylic acid-2,2'-bipyridine})]^{2+}$ .

FIG. 21 is the molecular structure for  $[\text{Ru}(4,7\text{-diphenyl-1,10-phenanthroline}(\text{SO}_3\text{Na})_2)_2(4\text{-methyl,4'-carboxylic acid-2,2'-bipyridine})]^{2+}$ .

5 FIG. 22 is the molecular structure for  $[\text{Ru}(2,2'\text{-bipyridyl})(1,10\text{-phenanthroline-9-isothiocyanate})]$ .

FIG. 23 is the molecular structure for  $[\text{Os}(1,10\text{-phenanthroline})_2(5\text{-amino-phenanthroline})]^{2+}$ .

10 FIG. 24 is the molecular structure for  $[\text{Os}(2,2':6',2''\text{-terpyridine})(\text{Bis}(2\text{-diphenylphosphinoethyl})\text{phenyl phosphine})]^{2+}$ .

FIG. 25 is the molecular structure for  $[\text{Ru bis}(2,2'\text{-bipyridyl})(\text{phenanthroline-maleamide})]$ .

FIG. 26 is the molecular structure for  $[\text{Os}(2,2'\text{-bipyridyl})_2(4,4'\text{-dicarboxylic acid-2,2'-bipyridine})][\text{PF}_6]_2$ .

15 FIG. 27 is the molecular structure for  $[\text{Os}(2,2'\text{-bipyridyl})_2(4,4'\text{-N-hydroxysuccidimide ester-2,2'-bipyridyl})][\text{PF}_6]_2$ .

20 FIG. 28 is a schematic of a solid state lifetime imager. The excitation can be an amplitude-modulated LED and the emission detected with a gatable CCD.

#### DETAILED DESCRIPTION OF THE INVENTION

25 In accordance with one embodiment of the present invention, a method is disclosed for detecting the presence of a nucleic acid sequence in a sample, by detecting the fluorescence of metal-ligand complexes.

In accordance with another embodiment of the present invention, a composition is disclosed which includes a

fluorescent metal-ligand complex, for detecting the presence of a nucleic acid sequence.

5 In accordance with another embodiment of the present invention, a diagnostic kit is disclosed which includes a fluorescent metal-ligand complex, for detecting the presence of a nucleic acid sequence.

10 The invention suggests the use of fluorescent metal-ligand complexes for *in situ* hybridization. The use of fluorescent metal-ligand complexes, as opposed to the commonly used organic probes such as fluorescein and rhodamine attached to DNA, solves the problem of low sensitivity based on the interfering fluorescence which occurs from all samples. Fluorescent metal-ligand complexes can provide an increased sensitivity because the long lifetimes will allow off-gating of the prompt autofluorescence.

15 In addition, all presently used fluorophores are unstable in the presence of continued illumination. Metal-ligand complexes according to the invention solve these problems by displaying long lifetimes, and are thus temporally resolved from the background fluorescence. The large Stoke's shift also allows higher sensitivity by an increased ability to filter out the interfering fluorescence. Furthermore, metal-ligand complexes according to the invention are highly photostable and thus allow sensitivity detection by imaging over a long period of time with continued illumination or with pulse elimination and gated detection.

There are a number of metal-ligand complexes which display luminescence, including complexes containing Co, Cr, Cu, Mo, Ru, Rh, W, Re, Os, Ir, or Pt. In particular, transition metal complexes, especially those with Ru, Os, Re, Rh, Ir, W or Pt, can be used. The metal in the metal-ligand complex is particularly preferably selected from the group consisting of ruthenium, osmium, and rhenium.

A suitable ligand in the metal-ligand complex can be polypyridine, bipyridine, or a related compound, and the ligand can contain a reactive group commonly used for linkage to biological molecules, such as a N-hydroxysuccinimide ester of a carboxylic acid, haloacetyl groups, maleimides, sulfonyl chlorides, and isothiocyanates. Other ligands for such metal-ligand complexes are bipyrazyl, phenanthroline, and related substituted derivatives, or inorganic ligands such as CO, Cl, nitrile and isonitrile. Table 1 shows that the lifetime can be varied by variation of the ligand attached to the metal.

20

Table 1

L	QY.	$\tau$ (ns)	$\lambda_{em\ max}$ (nm)
Cl-	0.005	51	622
4-NH <sub>2</sub> Py	0.052	129	597
4-EtPy	0.18	604	567
25 Py	0.16	669	558
P(CH <sub>3</sub> ) <sub>3</sub>	0.27	1169	544
CH <sub>3</sub> CN	0.41	1201	536

Preferred metal-ligand complexes include [Re(2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline)(CO)<sub>3</sub>(isonicotinic acid)]<sup>+</sup>, [Ru(4,7-diphenyl-1,10-phenanthroline)<sub>2</sub>(4,4'-dicarboxylic acid-2,2'-bipyridine)]<sup>2+</sup>, [Ru(4,7-diphenyl-1,10-phenanthroline)<sub>2</sub>(4-methyl,4'-carboxylic acid-2,2'-bipyridine)]<sup>2+</sup>, [Ru(4,7-diphenyl-1,10-phenanthroline)(SO<sub>3</sub>Na)<sub>2</sub>]<sub>2</sub>(4,4'-dicarboxylic acid-2,2'-bipyridine)]<sup>2+</sup>, [Ru(4,7-diphenyl-1,10-phenanthroline)(SO<sub>3</sub>Na)<sub>2</sub>]<sub>2</sub>(4-methyl,4'-carboxylic acid-2,2'-bipyridine)]<sup>2+</sup>, [Ru(2,2'-bipyridyl)(1,10-phenanthroline-9-isothiocyanate)], [Os(1,10-phenanthroline)<sub>2</sub>(5-amino-phenanthroline)]<sup>2+</sup>, [Os(2,2':6',2"-terpyridine)(Bis(2-diphenylphosphinoethyl)phenyl phosphine)]<sup>2+</sup>, [Ru bis(2,2'-bipyridyl)(phenanthroline-maleamide)], [Os(2,2'-bipyridyl)<sub>2</sub>(4,4'-dicarboxylic acid-2,2'-bipyridine)][PF<sub>6</sub>]<sub>2</sub>, [Os(2,2'-bipyridyl)<sub>2</sub>(4,4'-N-hydroxysuccidimide ester-2,2'-bipyridyl)][PF<sub>6</sub>]<sub>2</sub>, and [Ru(2,2'-bipyridyl)<sub>2</sub>(4,4'-N-hydroxysuccidimide ester-2,2'-bipyridyl)]<sup>2+</sup>.

Fluorescence *in situ* hybridization has become widely used in the medical sciences. The DNA to be tested, typically metaphase chromosomes, are exposed to probe DNA. The probe DNA has a base sequence directed toward one or more chromosomes. The probe DNA can be specific for the centromic or tetomeric region of chromosomes, or can be directed toward the entire chromosome. DNA probes can also be specific for small regions of DNA representing several genes. The exposure conditions result in

denaturation of the chromosomes and hybridization with the probe DNA.

*In situ* hybridization has been detected using radioactive tracers and autoradiography. For fluorescence  
5 *in situ* hybridization the probe DNA is made as fluorescent as possible by labeling with any fluorophore which does not interfere with hybridization. The reactive amino group attached to the base can be coupled to any of a wide variety of fluorescent probes. One aspect of the  
10 fluorescence *in situ* hybridization technology is that fluorophores can be incorporated with DNA without disruption of the base pairing. DNA can be synthesized with relatively high levels of such nucleotides.

Fluorescence *in situ* hybridization allows  
15 identification of all 23 human chromosomes. It is possible to develop DNA probes which paint each of the human chromosomes. Chromosome painting probes are developed by polymerase chain reaction (PCR) amplification of DNA fragments from chromosomes sorted by flow  
20 cytometry. Non-specific binding is prevented by adding additional DNA which binds to the most common sequences. However, the potential of fluorescence *in situ* hybridization for chromosome screening can only be realized if all the chromosomes can be simultaneously  
25 visualized. Unfortunately, 23 non-overlapping fluorophores are not presently available.

A variety of methods have been developed for multiple multiplex fluorescence *in situ* hybridization or spectra

karyotyping (SKY). Most methods include the use of multiple DNA probes each containing a different fluorophore. Differences in the chromosomes are revealed by the different colors or ratio of probes bound to each region of the chromosome. The most spectacular advances in fluorescence *in situ* hybridization have resulted from characterization of the emission spectra, using either an interferometric method or interference filters. By using just five colors for the chromosome paints it is possible to identify each of the 23 chromosomes. These are then assigned easily recognized pseudo colors. These advanced fluorescence *in situ* hybridization methods can provide a means to detect by gross and simple abnormalities, chromosome rearrangements, monitoring of bone marrow cells following transplantation and cancer chemotherapy.

Fluorescence *in situ* hybridization has many other important applications besides identifying chromosomes. By use of appropriate DNA sequences fluorescence *in situ* hybridization can be used to detect the human papillomavirus thought to be responsible for cervical cancer, and determination of fetal sex from amniotic fluid. Many of the applications of fluorescence *in situ* hybridization rely on computerized imaging and high sensitivity CCD detection. Fluorescence *in situ* hybridization technology represents a combination of modern optics, molecular biology and fluorescence spectroscopy.



The metal-ligand complexes of the present invention may be used in place of the known fluorophores in any suitable *in situ* hybridization method.

*In situ* hybridization has numerous applications.

5 Examples of uses for *in situ* hybridization include, but are not limited to, detection of human papillomavirus, detection of the messenger RNA for ovulation hormone, painting of chromosomes for identification of the chromosomes, detection of chromosome rearrangements,  
10 delineation of break points and chromosome abnormalities, cancer detection, detection of the gene for muscular dystrophy, and detection of sperm in cases of alleged sexual assault.

One embodiment of the present invention utilizes a  
15 metal-ligand complex which is capable of emitting polarized fluorescent light after being excited with linearly polarized electromagnetic light energy. A detailed description of this method is disclosed in U.S. Application Serial No. 08/330,743, incorporated herein by  
20 reference.

Another embodiment of the present invention utilizes fluorescent donor/acceptor pairs, or intercalating dyes, to detect DNA hybridization.

In another embodiment of the present invention, the  
25 release of the metal-ligand complex is quenched by an acceptor prior to cleavage of the probe. Such dequenching assays are used in PCR. A description of the prior PCR method is disclosed in Gibson, U.E.M., Heid, C.A., and

Williams, P.M., "A Novel Method for Real Time Quantitative RT-PCR," *Genome Research* 6:995-1001 (1996).

5 A further embodiment of the present invention utilizes selection of complexes with different lifetimes, so that the presence of the nucleic acid can be identified by the decay time of the label. Lifetime measurements are presently possible by measurements using either the time domain or the frequency domain, but the instrumentation is complex for nanosecond decay times. Alternately,  
10 fluorescence lifetime imaging of the gels which contain the labeled DNA can be utilized.

A description of a lifetime imaging apparatus is provided in U.S. patent 5,485,530, incorporated herein by reference.

15 Descriptions of the prior fluorescence lifetime methods are disclosed in the following references:

H. Szmazinski, J.R. Lakowicz, M.L. Johnson, "Fluorescence Lifetime Imaging Microscopy: Homodyne Technique Using High-Speed Gated Image Intensifier,"  
20 *Methods in Enzymology*, 240:723-748 (1994).

J.R. Lakowicz, H. Szmazinski, K. Nowaczyk, W.J. Lederer, M.S. Kirby, and M.L. Johnson, "Fluorescence lifetime imaging of intracellular calcium in COS cells using Quin-2," *Cell Calcium* 15:7-27 (1994).

25 J.R. Lakowicz, P.A. Koen, H. Szmazinski, I. Gryczynski, and J. Kusba, "Emerging Biomedical and Advanced Applications of Time-Resolved Fluorescence Spectroscopy," *Journal of Fluorescence* 4:117-136 (1994).

J.R. Lakowicz, H. Szmecinski, K. Nowaczyk, K.W. Berndt, and M. Johnson, "Fluorescence Lifetime Imaging," *Analytical Biochemistry* 202:316-330 (1992).

5 J.R. Lakowicz, H. Szmecinski, K. Nowaczyk, and M.L. Johnson, "Fluorescence lifetime imaging of free and protein-bound NADH," *Proc. Natl. Acad. Sci., USA* 89:1271-1275 (1992).

In another embodiment of the invention, capillary electrophoresis is used to determine the base sequence.  
10 Descriptions of prior capillary electrophoresis methods are disclosed in the following references:

J.A. Brumbaugh, L.R. Middendorf, D.L. Grone, and J.L. Ruth, "Continuous on-line DNA sequencing using oligodeoxynucleotide primers with multiple fluorophores,"  
15 *Proc. Natl. Acad. Sci. USA* 85:5610-14 (1988).

H. Swerdlow, J.Z. Zhang, D.Y. Chen, H.R. Harke, R. Grey, S. Wu, and N.J. Dovichi, "Three DNA Sequencing Methods Using Capillary Gel Electrophoresis and Laser-Induced Fluorescence," *Anal. Chem.* 63:2835-2841 (1991).

20 X.C. Huang, M.A. Quesada, and R.A. Mathies, "DNA Sequencing Using Capillary Array Electrophoresis," *Anal. Chem.*, 64:2149-2154 (1992).

S. Carson, A.S. Cohen, A. Belenkii, M.C. Ruiz-Martinez, J. Berka, and B.L. Karger, "DNA Sequencing by  
25 Capillary Electrophoresis: Use of a Two-Laser-Two-Window Intensified Diode Array Detection System," *Anal. Chem.* 65:3219-3226 (1993).

K. Ueno and E.S. Yeung, "Simultaneous Monitoring of DNA Fragments Separated By Electrophoresis in a Multiplexed Array of 100 Capillaries," *Anal. Chem.*, 66: 1424-1431 (1994).

- 5 L.R. Middendorf, J.A. Brumbaugh, D.L. Grone, C.A. Morgan, and J.L. Ruth, "Large scale DNA sequencing," *American Biotechnology Laboratory*, August 1988.

DNA chip technology, which utilizes light directed matrices, may be used in another embodiment of the present invention. This technology is available in Affymetrix's  
10 GeneChip™ and Hyseq's SuperChips™.

Descriptions of prior DNA chip technology are disclosed in the following references:

- S.P.A. Fodor, "Massively Parallel Genomics," *Science*  
15 277:393-395 (1997).

A. Caviani Pease, D. Solas, E.J. Sullivan, M.T. Cronin, C.P. Holmes, and S.P.A. Fodor, "Light-generated oligonucleotide arrays for rapid DNA sequence analysis," *Proc. Natl. Acad. Sci. USA* 91:5022-5026 (1994).

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M.T. Cronin, R.V. Fucini, S.M. Kim, R.S. Masino, R.M. Wespi, and C.G. Miyada, "Cystic Fibrosis Mutation  
25 Detection by Hybridization to Light-Generated DNA Probe Arrays," *Human Mutation* 7:244-255 (1996).

The capillary tubes or the DNA chips are imaged using CCD technology and the gain modulated image intensifier. This techniques allows the decay times to be measured at each point in the image, without single spot pixel by pixel scanning. A self-gated CCD camera, which does not have an image intensifier, can be used with long-lived metal ligand complexes. The use of a self-gated CCD camera is simpler than using an image intensifier. Long-lifetime metal ligand complexes are ideal for use with CCD cameras, which have relatively slow speeds (Figure 28).

The invention is further illustrated by the following examples, which are not intended to be limiting.

Example 1:

A fluorescent metal-ligand complex having the formula  $[\text{Ru}(2,2'\text{-bipyridyl})_2(4,4'\text{-N-hydroxysuccidimide ester-2,2'\text{-bipyridyl})^{2+}]$  (Figure 9) is coupled to an oligonucleotide containing a reactive amine to form a probe. The amount of metal-ligand complex is about ten-fold excess over the amount of oligonucleotide, and the reaction is carried out under basic conditions near pH 8, using a carbonate buffer which is not nucleophilic and does not react with the metal-ligand complex probe. Following purification by any suitable known procedures, the metal-ligand complex probe is added to a sample believed to contain a nucleic acid sequence complimentary to the oligonucleotide sequence of the probe, and the

reaction product is formed. The mixture is exposed  
to radiation near 450 nm, and a long pass optical  
filter is utilized to detect the emission near 600  
nm. Detection can be performed as a steady state  
5 measurement, or with time-gated detection with off-  
gating of the autofluorescence and on-gating of the  
long-lived metal-ligand complex fluorescence.  
Binding may also be detected by fluorescence  
polarization, in which case the excitation of the  
10 emission beams will contain polarizers.

Since many modifications, variations, and changes in  
detail may be made to the described embodiments, it is  
intended that all matter in the foregoing description and  
shown in the accompanying drawings be interpreted as  
15 illustrative and not in a limiting sense.

CLAIMS

1. A method for detecting the presence of a nucleic acid sequence in a sample comprising the steps of:

coupling a fluorescent metal-ligand complex to an oligonucleotide having a sequence complementary to said nucleic acid sequence to form a probe;

5 adding said probe to a sample that contains said nucleic acid sequence to form a mixture containing a reaction product;

10 exposing said mixture to an exciting amount of radiation;

detecting fluorescence of said metal-ligand complex; and

15 determining presence of said nucleic acid sequence based on fluorescence of said metal-ligand complex.

2. A method as defined by claim 1, wherein said nucleic acid indicates the presence of human papillomavirus.

3. A method as defined by claim 1, wherein said nucleic acid sequence is present in a chromosome.

4. A method as defined by claim 1, wherein said nucleic acid sequence indicates a chromosome rearrangement.

5. A method as defined by claim 1, wherein said nucleic acid sequence indicates the presence of chromosome break points and abnormalities.

6. A method as defined by claim 1, wherein said nucleic acid sequence indicates the presence of cancer.

7. A method as defined by claim 1, wherein said nucleic acid sequence indicates the presence of sperm.

8. A method as defined by claim 1, wherein said nucleic acid sequence is messenger RNA for ovulation hormone.

9. A method as defined by claim 1, wherein said nucleic acid sequence is present in a gene coding for muscular dystrophy.

10. A method as defined by claim 1, wherein the metal in said fluorescent metal-ligand complex is a transition metal.

11. A method as defined by claim 1, wherein the metal in said fluorescent metal-ligand complex is selected from the group consisting of Co, Cr, Cu, Mo, Ru, Rh, W, Re, Os, Ir, and Pt.



12. A method as defined by claim 1, wherein the metal in said fluorescent metal-ligand complex is selected from the group consisting of Ru, Os, Re, Rh, Ir, W, and Pt.

13. A method as defined by claim 1, wherein the metal in said fluorescent metal-ligand complex is selected from the group consisting of Ru, Os, and Rh.

14. A method as defined by claim 1, wherein said fluorescent metal ligand complex is selected from the group consisting of [Re(2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline)(CO)<sub>3</sub>(isonicotinic acid)]<sup>+</sup>, [Ru(4,7-diphenyl-1,10-phenanthroline)<sub>2</sub>(4,4'-dicarboxylic acid-2,2'-bipyridine)]<sup>2+</sup>, [Ru(4,7-diphenyl-1,10-phenanthroline)<sub>2</sub>(4-methyl,4'-carboxylic acid-2,2'-bipyridine)]<sup>2+</sup>, [Ru(4,7-diphenyl-1,10-phenanthroline)(SO<sub>3</sub>Na)<sub>2</sub>]<sub>2</sub>(4,4'-dicarboxylic acid-2,2'-bipyridine)]<sup>2+</sup>, [Ru(4,7-diphenyl-1,10-phenanthroline)(SO<sub>3</sub>Na)<sub>2</sub>]<sub>2</sub>(4-methyl,4'-carboxylic acid-2,2'-bipyridine)]<sup>2+</sup>, [Ru(2,2'-bipyridyl)(1,10-phenanthroline-9-isothiocyanate)], [Os(1,10-phenanthroline)<sub>2</sub>(5-amino-phenanthroline)]<sup>2+</sup>, [Os(2,2':6',2"-terpyridine)(Bis(2-diphenylphosphinoethyl)phenyl phosphine)]<sup>2+</sup>, [Ru bis(2,2'-bipyridyl)(phenanthroline-maleamide)], [Os(2,2'-bipyridyl)<sub>2</sub>(4,4'-dicarboxylic acid-2,2'-bipyridine)][PF<sub>6</sub>]<sub>2</sub>, [Os(2,2'-bipyridyl)<sub>2</sub>(4,4'-N-hydroxysuccidimide ester

20      -2,2'-bipyridyl)][PF<sub>6</sub>]<sub>2</sub>, and [Ru(2,2'-bipyridyl)<sub>2</sub> (4,4'-N-hydroxysuccidimide ester-2,2'-bipyridyl)]<sup>2+</sup>.

15. A method as defined by claim 1, wherein autofluorescence is suppressed by fluorescence gating.

16. A method as defined by claim 1, wherein said nucleic acid sequence is detected using capillary electrophoresis.

17. A method as defined by claim 1, wherein said detection utilizes measurement of fluorescence lifetime.

18. A method as defined by claim 1,

5            wherein said metal-ligand complex is capable of emitting polarized fluorescent light after being excited with linearly polarized electromagnetic light energy;

          wherein said radiation is linearly polarized electromagnetic light energy which causes said mixture to emit polarized fluorescent light;

10            wherein said detection is measurement of polarization of fluorescent light emission;

          wherein prior to said coupling step, said oligonucleotide is exposed to said linearly polarized electromagnetic light energy and polarization of fluorescent light is measured; and

15                wherein said determination of presence of said nucleic acid utilizes the change in polarization of fluorescent light emission of the oligonucleotide and polarization of fluorescent light emission of the mixture.

19. A composition for detecting presence of a nucleic acid sequence, comprising a fluorescent metal-ligand complex coupled to an oligonucleotide having a sequence complementary to said nucleic acid sequence.

20. A composition as defined by claim 19, wherein said nucleic acid sequence is present in human papillomavirus.

21. A composition as defined by claim 19, wherein said nucleic acid sequence is present in a chromosome.

22. A composition as defined by claim 19, wherein said nucleic acid sequence indicates presence of chromosome rearrangements.

23. A composition as defined by claim 19, wherein said nucleic acid sequence indicates presence of chromosome break points and abnormalities.

24. A composition as defined by claim 19, wherein said nucleic acid sequence indicates presence of cancer.

25. A composition as defined by claim 19, wherein said nucleic acid sequence indicates presence of sperm.

26. A composition as defined by claim 19, wherein said nucleic acid sequence is messenger RNA for ovulation hormone.

27. A composition as defined by claim 19, wherein said nucleic acid sequence is a gene coding for muscular dystrophy.

28. A composition as defined by claim 19, wherein the metal in said fluorescent metal-ligand complex is a transition metal.

29. A composition as defined by claim 19, wherein the metal in said fluorescent metal-ligand complex is selected from the group consisting of Co, Cr, Cu, Mo, Ru, Rh, W, Re, Os, Ir, or Pt.

30. A composition as defined by claim 19, wherein the metal in said fluorescent metal-ligand complex is selected from the group consisting of Ru, Os, Re, Rh, Ir, W, or Pt.

31. A composition as defined by claim 19, wherein the metal in said fluorescent metal-ligand complex is selected from the group consisting of Ru, Os, or Rh.

32. A composition as defined by claim 19, wherein said fluorescent metal ligand complex is selected from the group consisting of [Re(2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline)(CO)<sub>3</sub>(isonicotinic acid)]<sup>+</sup>, [Ru(4,7-diphenyl-1,10-phenanthroline)<sub>2</sub>(4,4'-dicarboxylic acid-2,2'-bipyridine)]<sup>2+</sup>, [Ru(4,7-diphenyl-1,10-phenanthroline)<sub>2</sub>(4-methyl,4'-carboxylic acid-2,2'-bipyridine)]<sup>2+</sup>, [Ru(4,7-diphenyl-1,10-phenanthroline(SO<sub>3</sub>Na)<sub>2</sub>)<sub>2</sub>(4,4'-dicarboxylic acid-2,2'-bipyridine)]<sup>2+</sup>, [Ru(4,7-diphenyl-1,10-phenanthroline(SO<sub>3</sub>Na)<sub>2</sub>)<sub>2</sub>(4-methyl,4'-carboxylic acid-2,2'-bipyridine)]<sup>2+</sup>, [Ru(2,2'-bipyridyl)(1,10-phenanthroline-9-isothiocyanate)], [Os(1,10-phenanthroline)<sub>2</sub>(5-amino-phenanthroline)]<sup>2+</sup>, [Os(2,2':6',2"-terpyridine)(Bis(2-diphenylphosphinoethyl)phenyl phosphine)]<sup>2+</sup>, [Ru bis(2,2'-bipyridyl)(phenanthroline-maleamide)], [Os(2,2'-bipyridyl)<sub>2</sub>(4,4'-dicarboxylic acid-2,2'-bipyridine)][PF<sub>6</sub>]<sub>2</sub>, [Os(2,2'-bipyridyl)<sub>2</sub>(4,4'-N-hydroxysuccidimide ester-2,2'-bipyridyl)][PF<sub>6</sub>]<sub>2</sub>, and [Ru(2,2'-bipyridyl)<sub>2</sub>(4,4'-N-hydroxysuccidimide ester-2,2'-bipyridyl)]<sup>2+</sup>.

33. A diagnostic kit for detecting presence of a nucleic acid sequence, comprising a fluorescent metal-ligand complex coupled to an oligonucleotide having a sequence complementary to said nucleic acid sequence.

## AMENDED CLAIMS

[received by the International Bureau on 24 May 1999 (24.05.99);  
original claims 1,9,18 and 29-31 amended; new claim 34 added;  
remaining claims unchanged 5 pages)]

1. A method for detecting the presence of a  
nucleic acid sequence in a sample comprising the steps  
of:

5           adding a probe labeled with a fluorescent  
metal-ligand complex to a sample that contains  
said nucleic acid sequence to form a mixture  
containing a reaction product;  
          exposing said mixture to an exciting amount  
10   of radiation;  
          detecting fluorescence of said metal-ligand  
complex; and  
          determining presence of said nucleic acid  
sequence based on fluorescence of said metal-  
15   ligand complex.

2. A method as defined by claim 1, wherein said  
nucleic acid indicates the presence of human  
papillomavirus.

3. A method as defined by claim 1, wherein said  
20   nucleic acid sequence is present in a chromosome.

4. A method as defined by claim 1, wherein said  
nucleic acid sequence indicates a chromosome  
rearrangement.

5. A method as defined by claim 1, wherein said nucleic acid sequence indicates the presence of chromosome break points and abnormalities.

6. A method as defined by claim 1, wherein said  
5 nucleic acid sequence indicates the presence of cancer.

7. A method as defined by claim 1, wherein said nucleic acid sequence indicates the presence of sperm.

8. A method as defined by claim 1, wherein said nucleic acid sequence is messenger RNA for ovulation  
10 hormone.

9. A method as defined by claim 1, wherein said nucleic acid sequence indicates the presence of muscular dystrophy.

10. A method as defined by claim 1, wherein the  
15 metal in said fluorescent metal-ligand complex is a transition metal.

11. A method as defined by claim 1, wherein the metal in said fluorescent metal-ligand complex is selected from the group consisting of Co, Cr, Cu, Mo,  
20 Ru, Rh, W, Re, Os, Ir, and Pt.

-2,2'-bipyridyl)][PF<sub>6</sub>]<sub>2</sub>, and [Ru(2,2'-bipyridyl)<sub>2</sub> (4,4'-N-hydroxysuccidimide ester-2,2'-bipyridyl)]<sup>2+</sup>.

15. A method as defined by claim 1, wherein autofluorescence is suppressed by fluorescence gating.

5           16. A method as defined by claim 1, wherein said nucleic acid sequence is detected using capillary electrophoresis.

10           17. A method as defined by claim 1, wherein said detection utilizes measurement of fluorescence lifetime.

15           18. A method as defined by claim 1,  
              wherein said metal-ligand complex is capable of emitting polarized fluorescent light after being excited with linearly polarized electromagnetic light energy;

              wherein said radiation is linearly polarized electromagnetic light energy which causes said mixture to emit polarized fluorescent light;

20           wherein said detection is measurement of polarization of fluorescent light emission;

              wherein prior to forming said reaction product, said oligonucleotide is exposed to said linearly polarized electromagnetic light energy and polarization of fluorescent light is measured;  
25           and



25. A composition as defined by claim 19, wherein said nucleic acid sequence indicates presence of sperm.

26. A composition as defined by claim 19, wherein said nucleic acid sequence is messenger RNA for ovulation hormone.

27. A composition as defined by claim 19, wherein said nucleic acid sequence indicates the presence of muscular dystrophy.

28. A composition as defined by claim 19, wherein the metal in said fluorescent metal-ligand complex is a transition metal.

29. A composition as defined by claim 19, wherein the metal in said fluorescent metal-ligand complex is selected from the group consisting of Co, Cr, Cu, Mo, Ru, Rh, W, Re, Os, Ir, and Pt.

30. A composition as defined by claim 19, wherein the metal in said fluorescent metal-ligand complex is selected from the group consisting of Ru, Os, Re, Rh, Ir, W, and Pt.

31. A composition as defined by claim 19, wherein the metal in said fluorescent metal-ligand complex is selected from the group consisting of Ru, Os, and Rh.

32. A composition as defined by claim 19, wherein said fluorescent metal ligand complex is selected from the group consisting of [Re(2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline)(CO)<sub>3</sub>(isonicotinic acid)]<sup>+</sup>, [Ru(4,7-  
 5 diphenyl-1,10-phenanthroline)<sub>2</sub>(4,4'-dicarboxylic acid-2,2'-bipyridine)]<sup>2+</sup>, [Ru(4,7-diphenyl-1,10-phenanthroline)<sub>2</sub>(4-methyl,4'-carboxylic acid-2,2'-bipyridine)]<sup>2+</sup>, [Ru(4,7-diphenyl-1,10-phenanthroline)(SO<sub>3</sub>Na)<sub>2</sub>]<sub>2</sub>(4,4'-dicarboxylic acid-2,2'-  
 10 bipyridine)]<sup>2+</sup>, [Ru(4,7-diphenyl-1,10-phenanthroline)(SO<sub>3</sub>Na)<sub>2</sub>]<sub>2</sub>(4-methyl,4'-carboxylic acid-2,2'-bipyridine)]<sup>2+</sup>, [Ru(2,2'-bipyridyl)(1,10-phenanthroline-9-isothiocyanate)], [Os(1,10-phenanthroline)<sub>2</sub>(5-amino-phenanthroline)]<sup>2+</sup>,  
 15 [Os(2,2':6',2"-terpyridine)(Bis(2-diphenylphosphinoethyl)phenyl phosphine)]<sup>2+</sup>, [Ru bis(2,2'-bipyridyl)(phenanthroline-maleamide)], [Os(2,2'-bipyridyl)<sub>2</sub>(4,4'-dicarboxylic acid-2,2'-bipyridine)][PF<sub>6</sub>]<sub>2</sub>, [Os(2,2'-bipyridyl)<sub>2</sub>(4,4'-N-hydroxysuccidimide ester-2,2'-bipyridyl)][PF<sub>6</sub>]<sub>2</sub>, and  
 20 [Ru(2,2'-bipyridyl)<sub>2</sub>(4,4'-N-hydroxysuccidimide ester-2,2'-bipyridyl)]<sup>2+</sup>.

33. A diagnostic kit for detecting presence of a nucleic acid sequence, comprising a fluorescent metal-  
 25 ligand complex coupled to an oligonucleotide having a sequence complementary to said nucleic acid sequence.

34. A method as defined by claim 1, which further comprises coupling said fluorescent metal-ligand complex to said oligonucleotide.

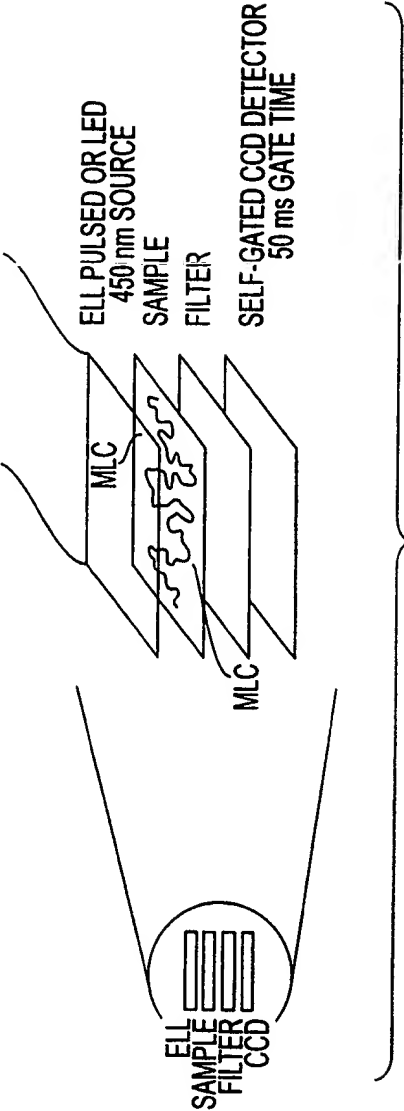


FIG. 1

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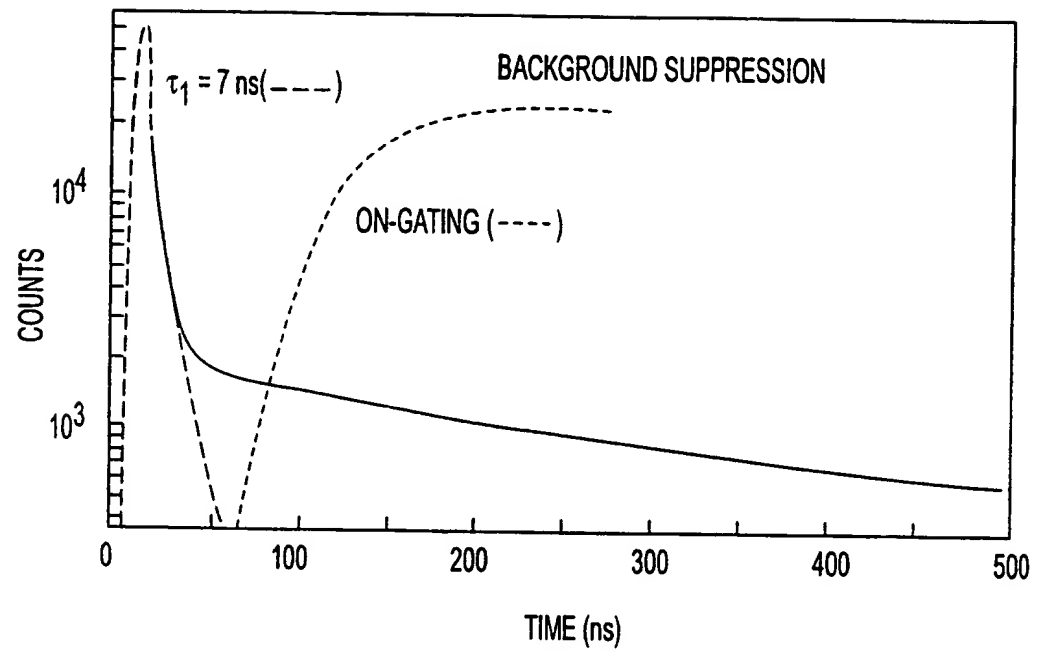


FIG. 2

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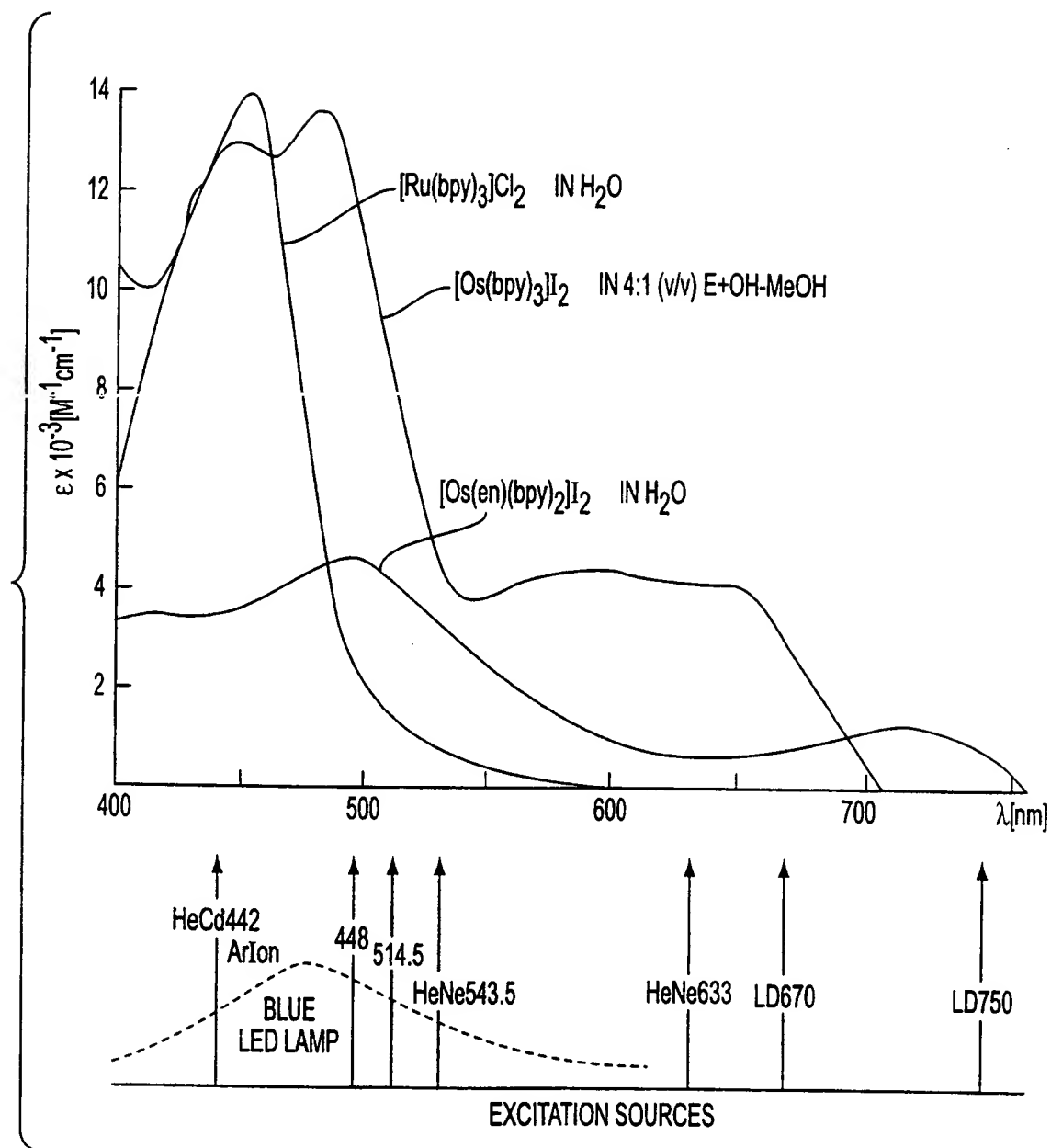


FIG. 3

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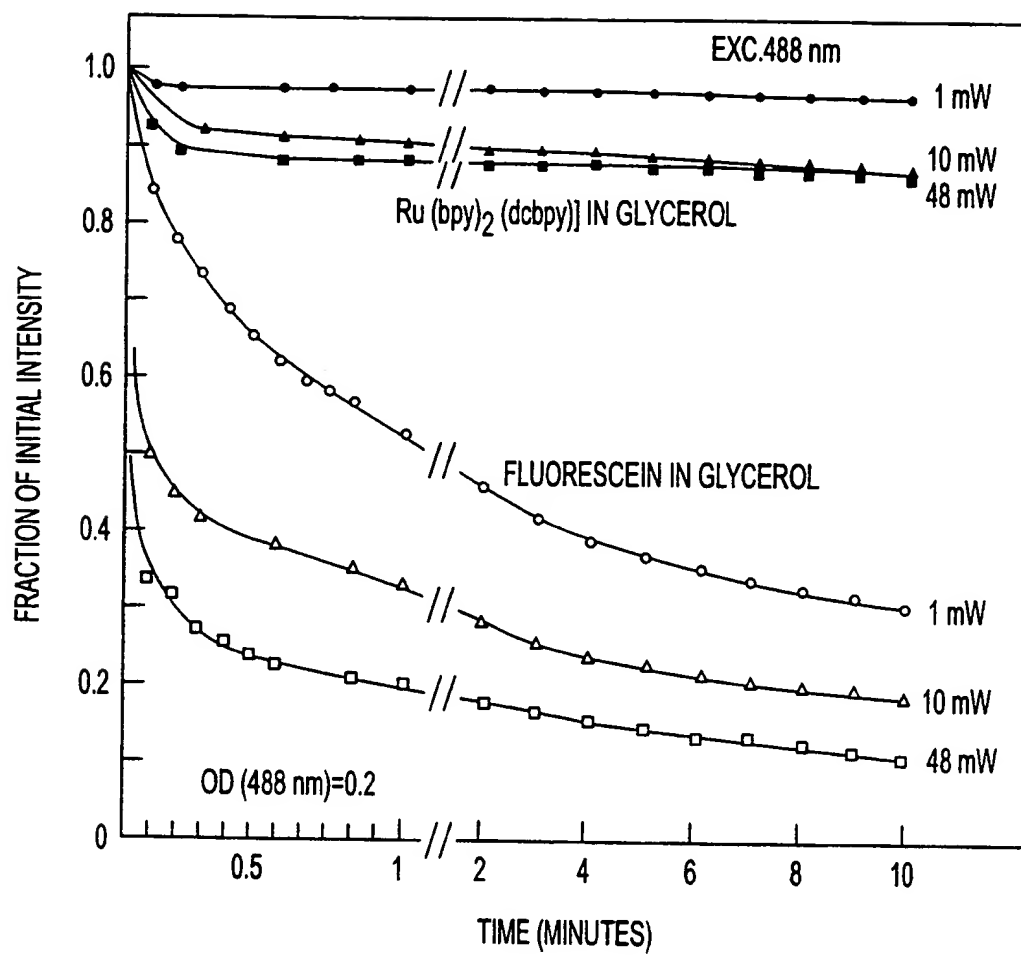


FIG. 4

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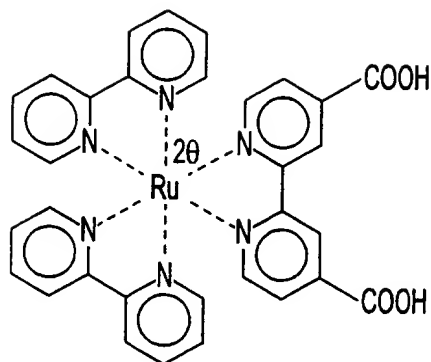


FIG. 5A

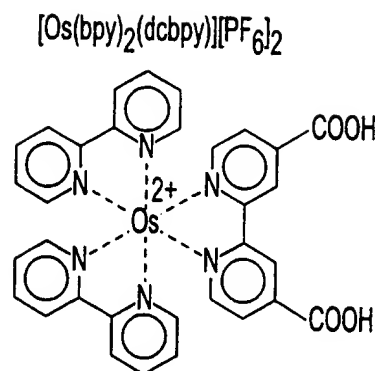


FIG. 5B

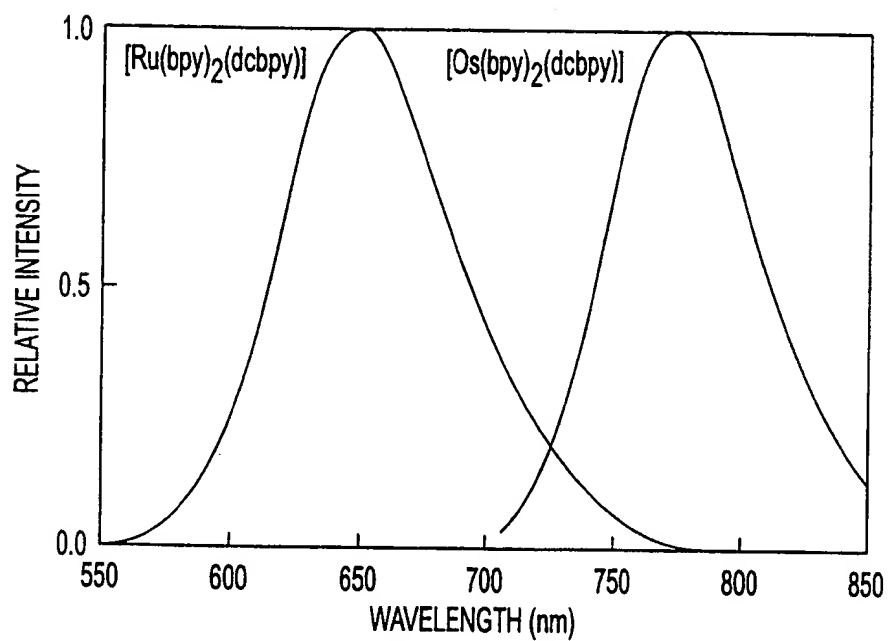


FIG. 5C

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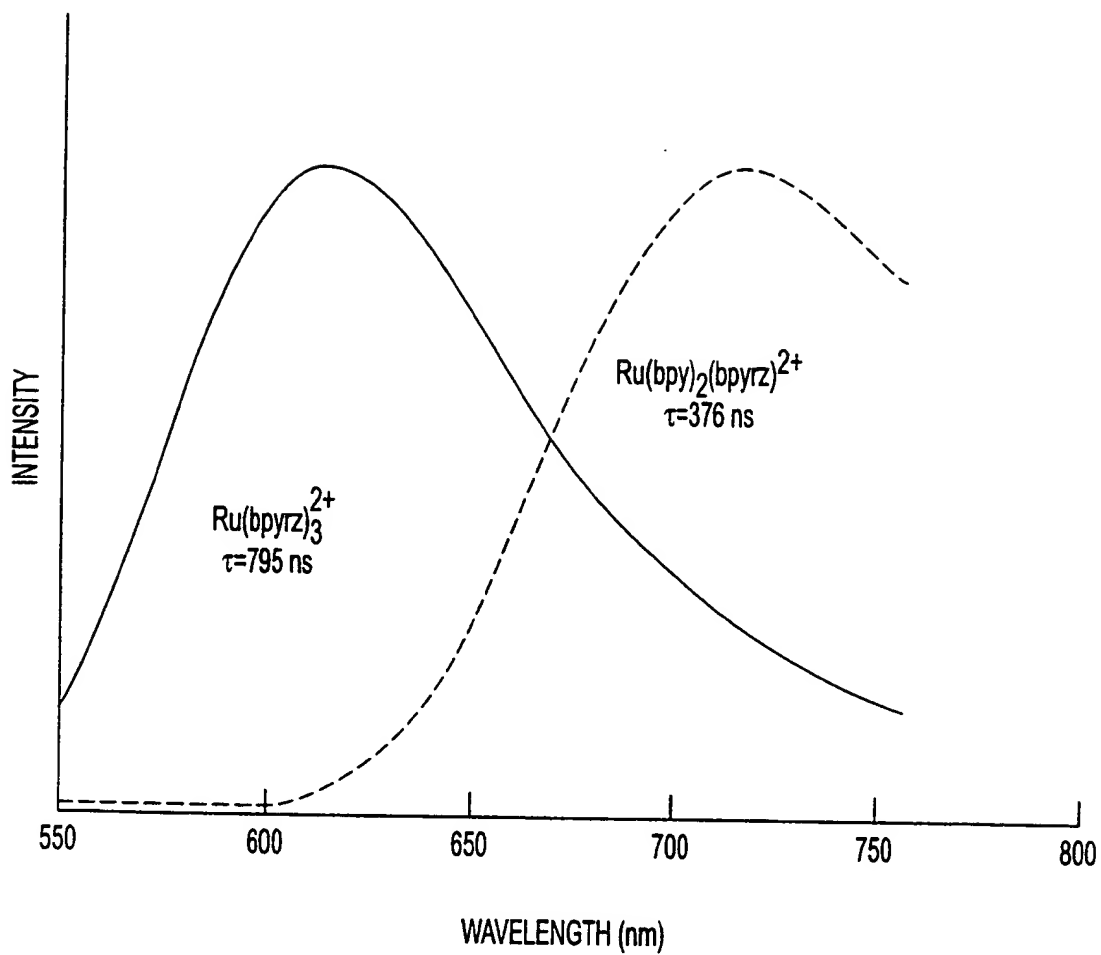


FIG. 6



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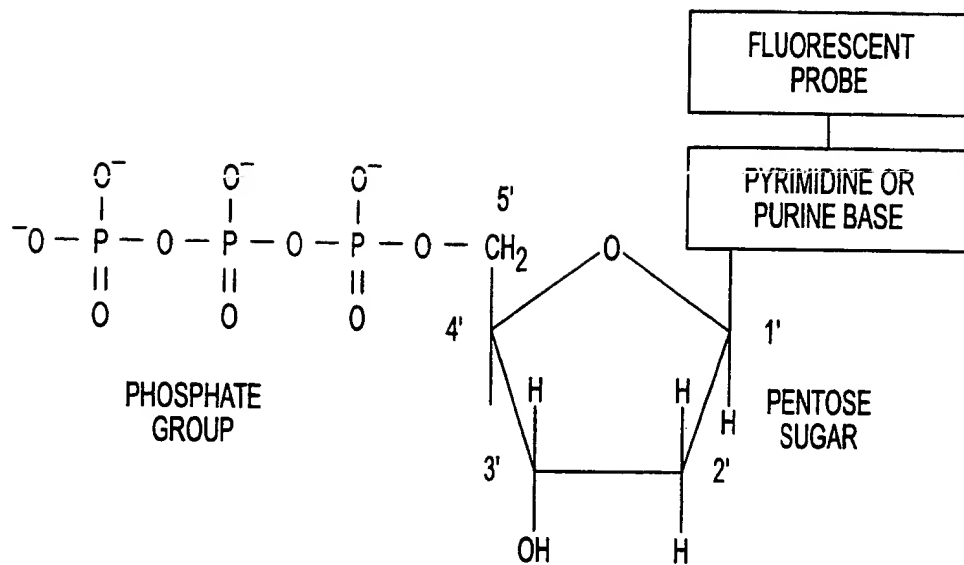


FIG. 7

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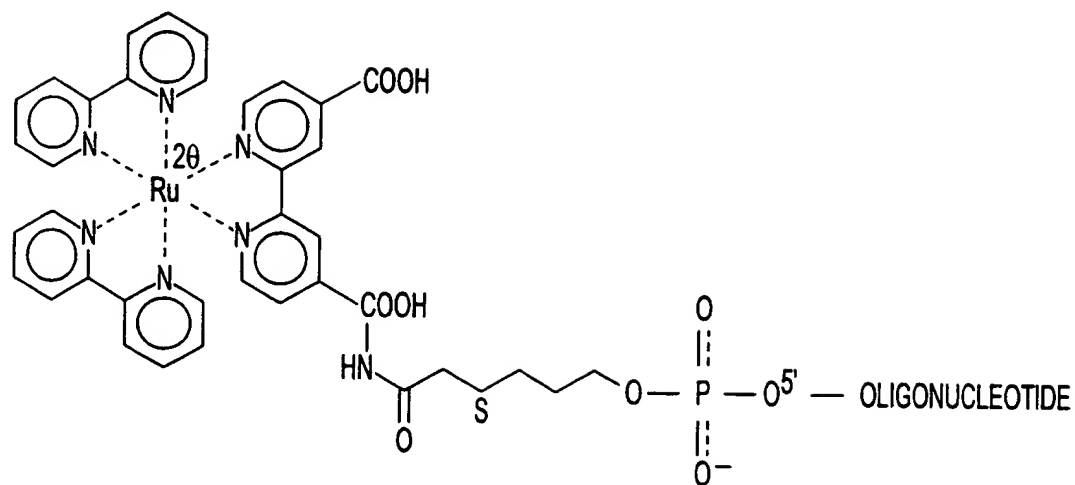


FIG. 8A

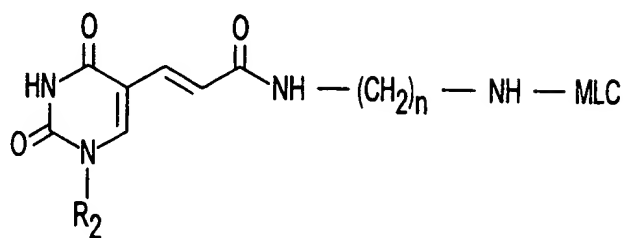


FIG. 8B

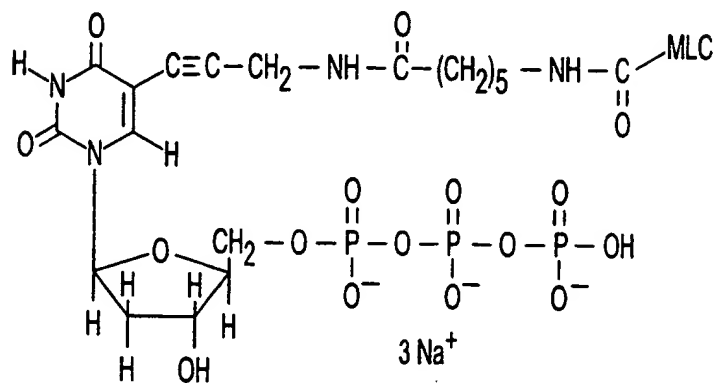


FIG. 8C

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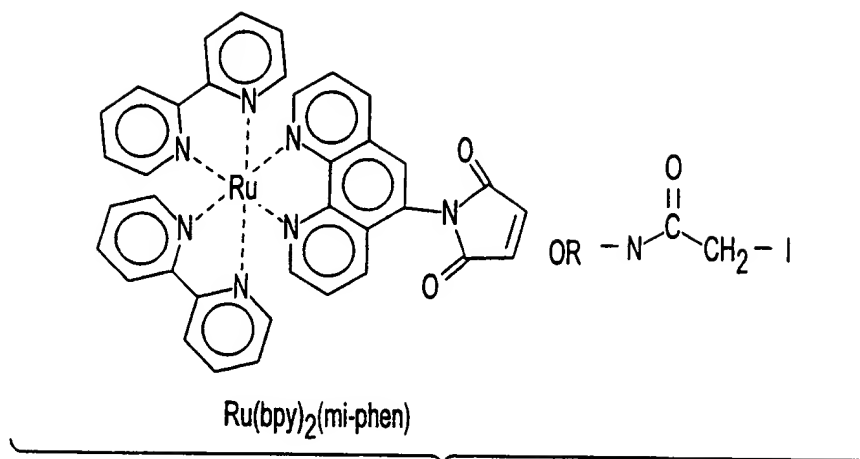


FIG. 9A

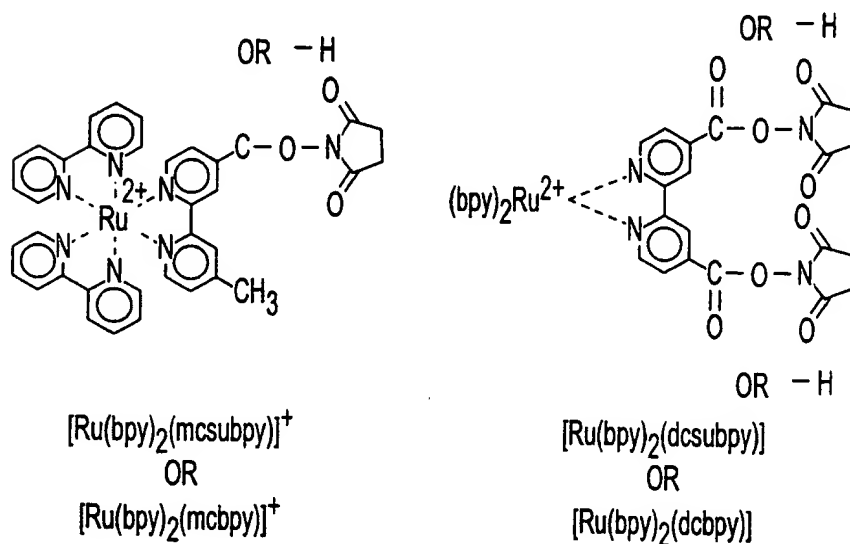


FIG. 9B

FIG. 9C

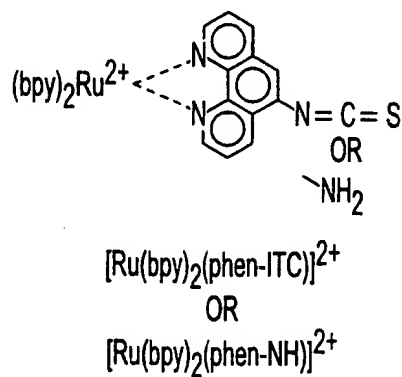


FIG. 9D

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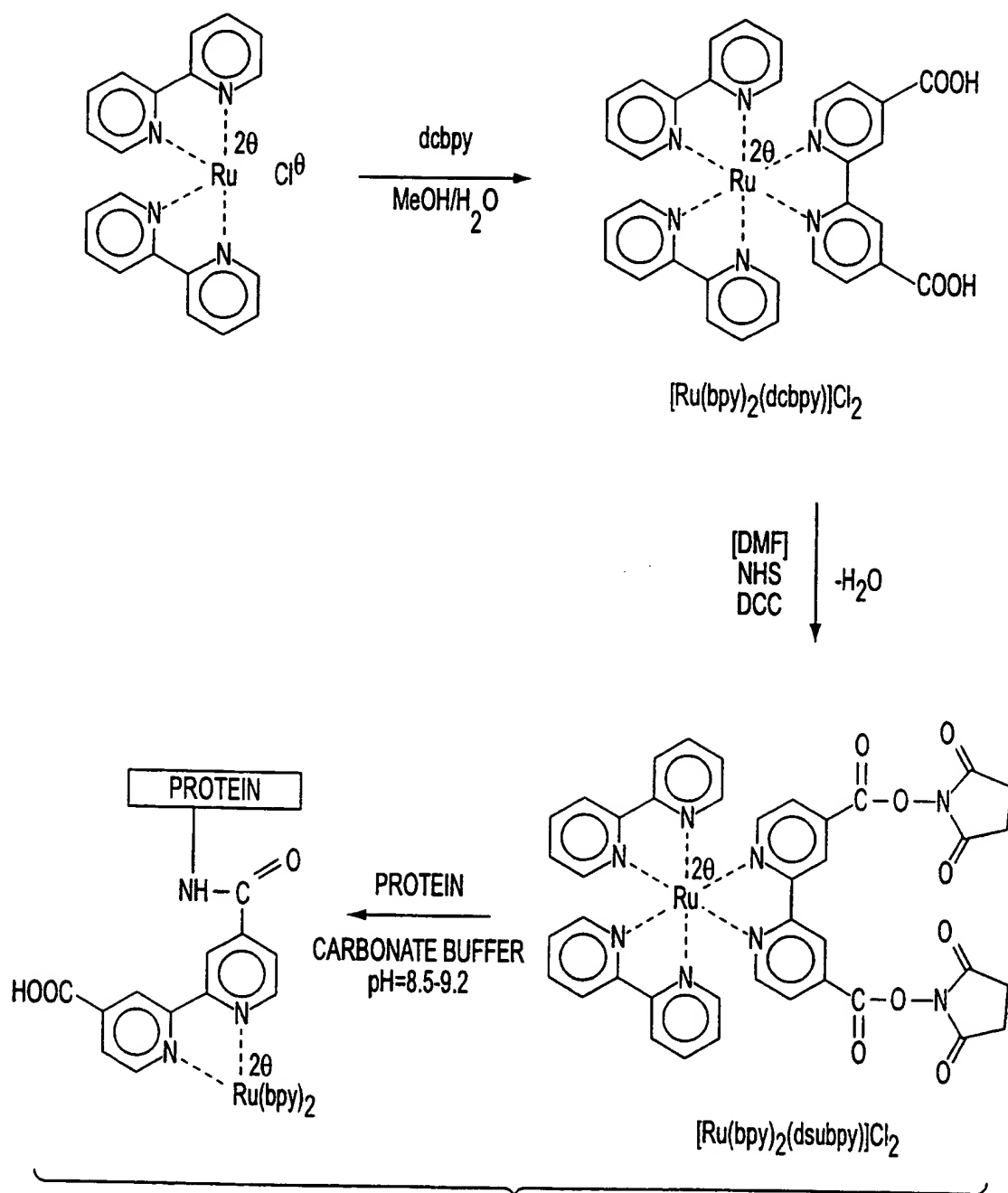


FIG. 10

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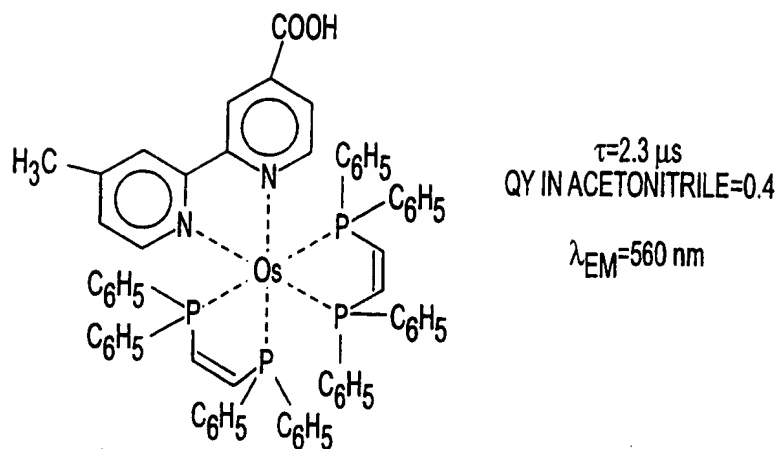


FIG. 11A

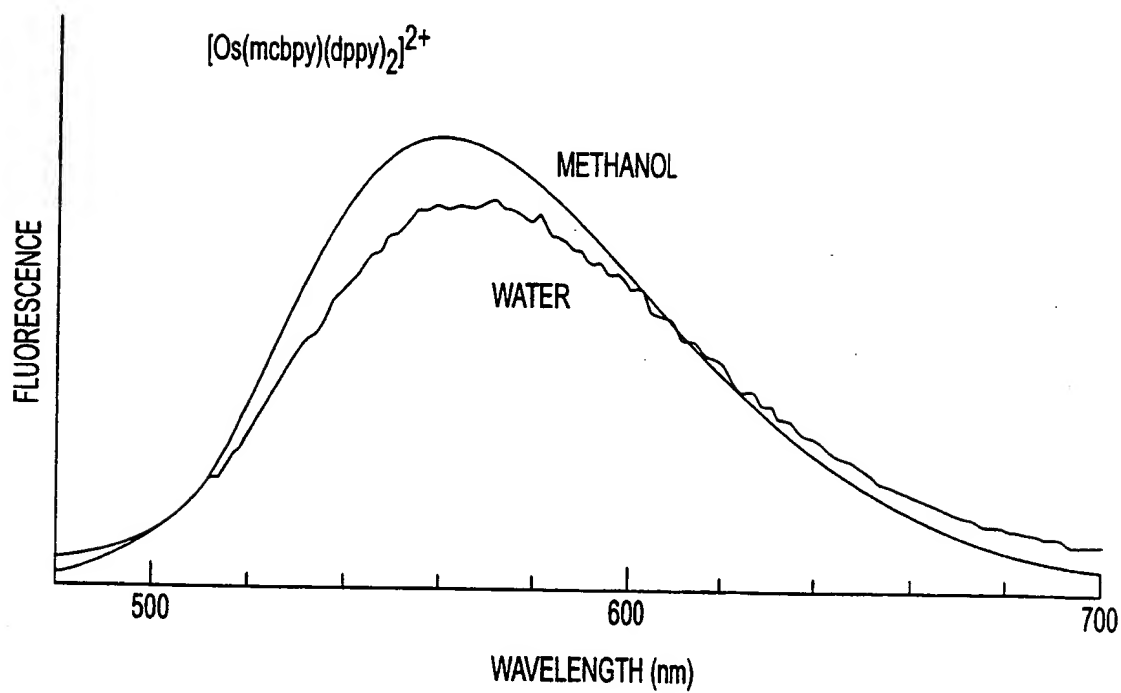


FIG. 11B

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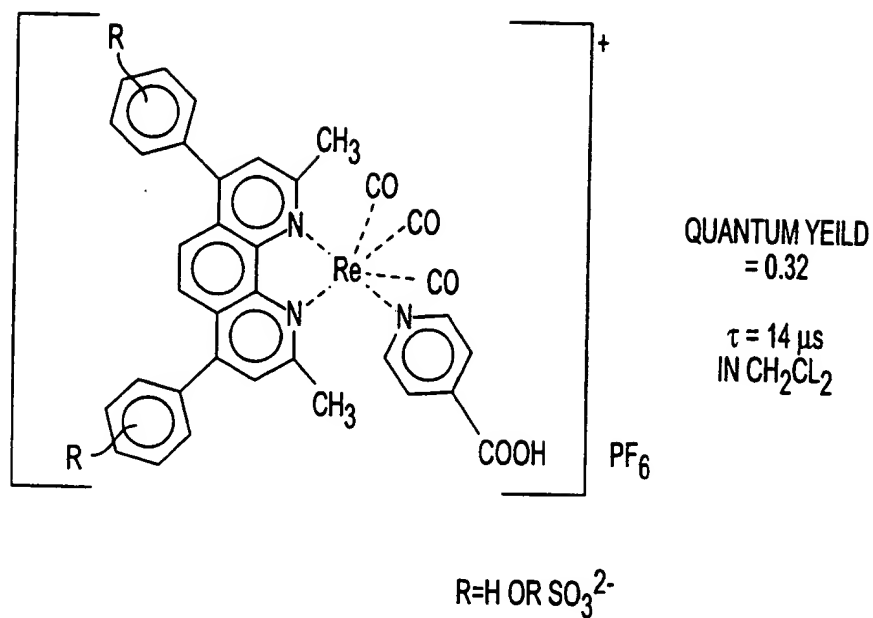


FIG. 12A

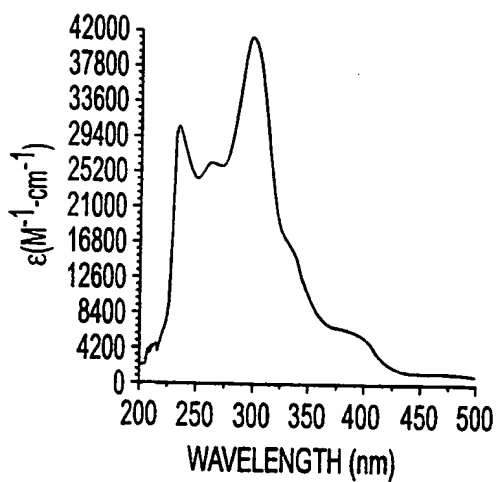


FIG. 12B

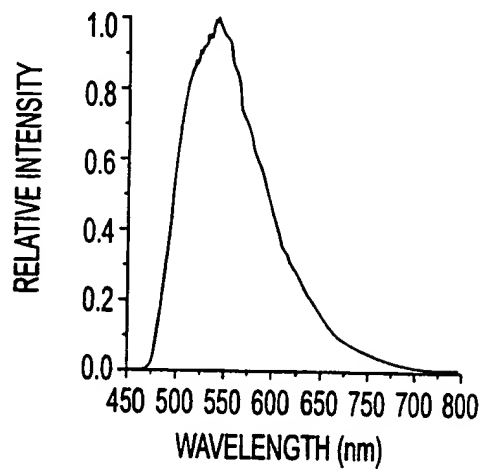


FIG. 12C

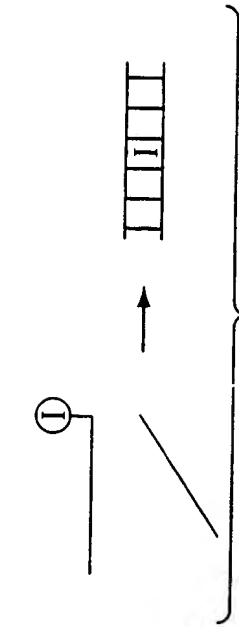


FIG. 13D

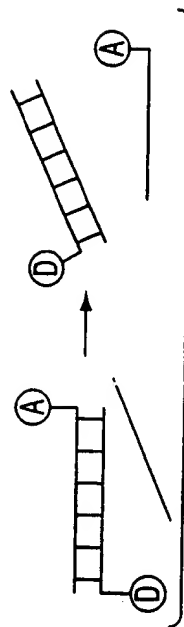


FIG. 13E

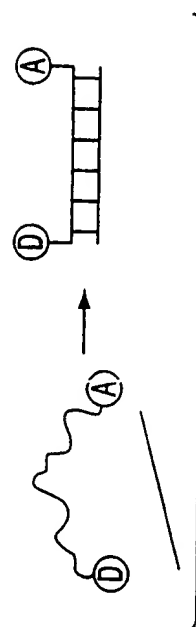


FIG. 13F

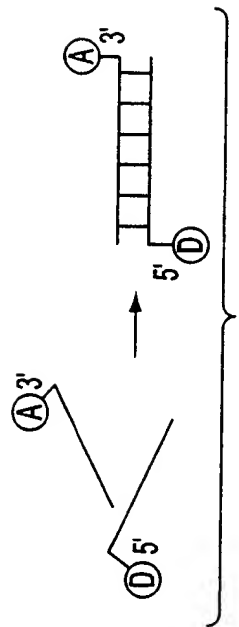


FIG. 13A

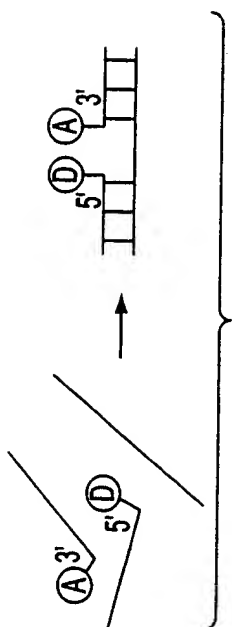


FIG. 13B

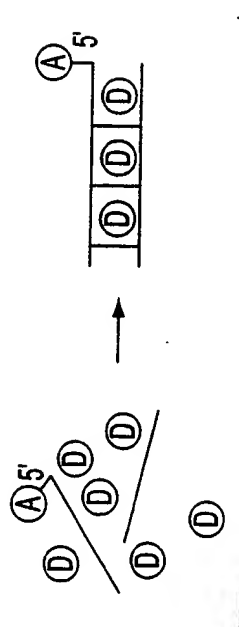


FIG. 13C

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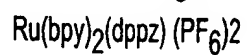
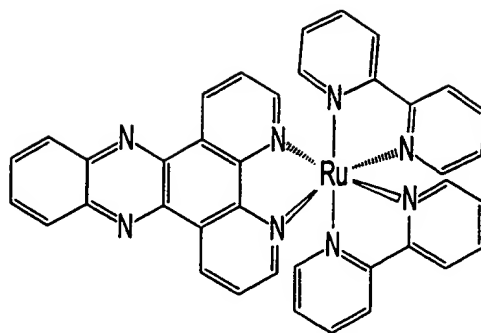


FIG. 14A

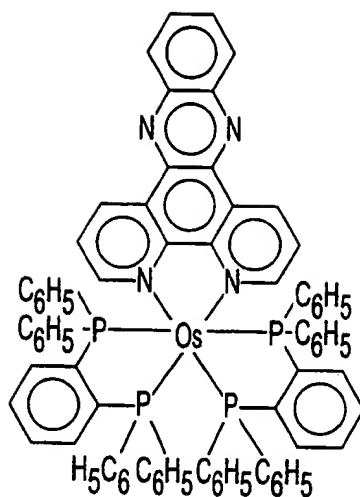


FIG. 14B

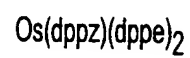
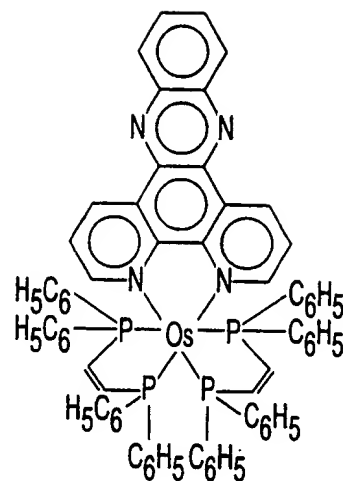
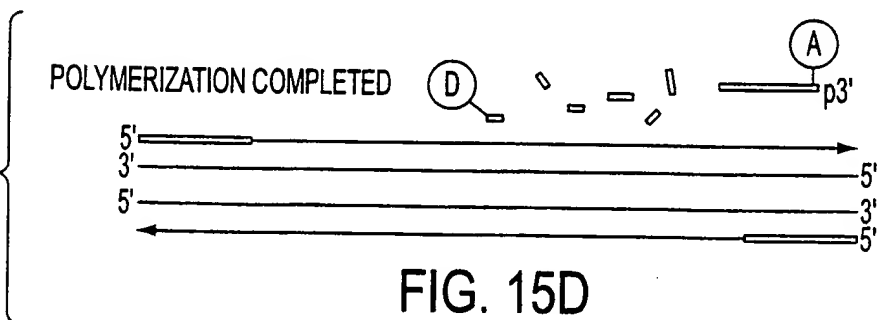
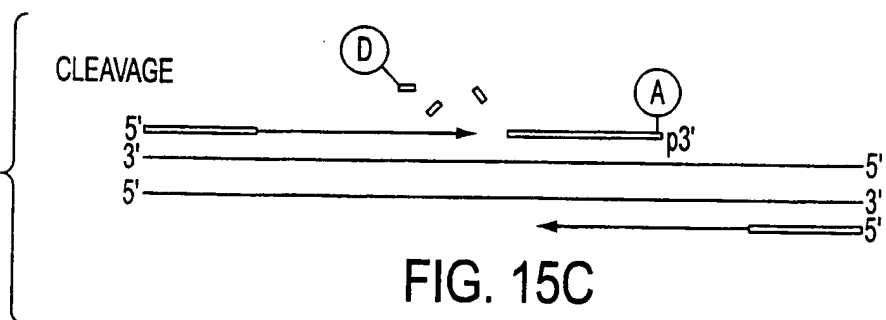
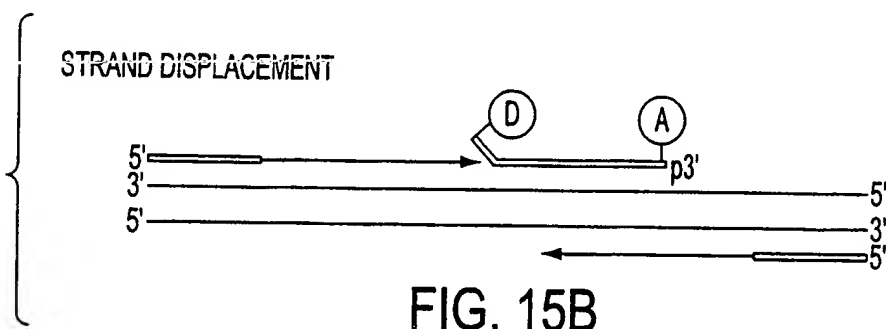
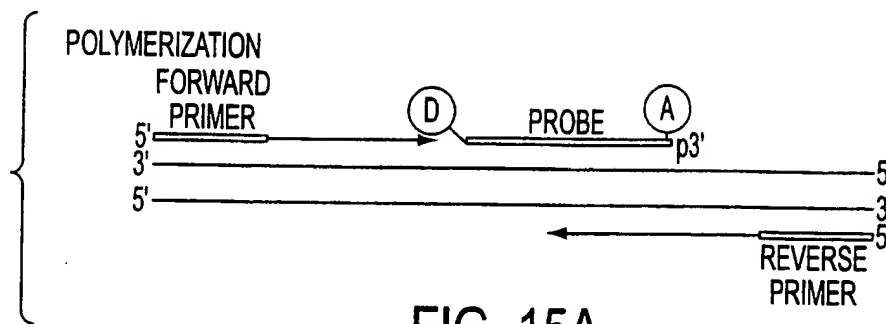


FIG. 14C



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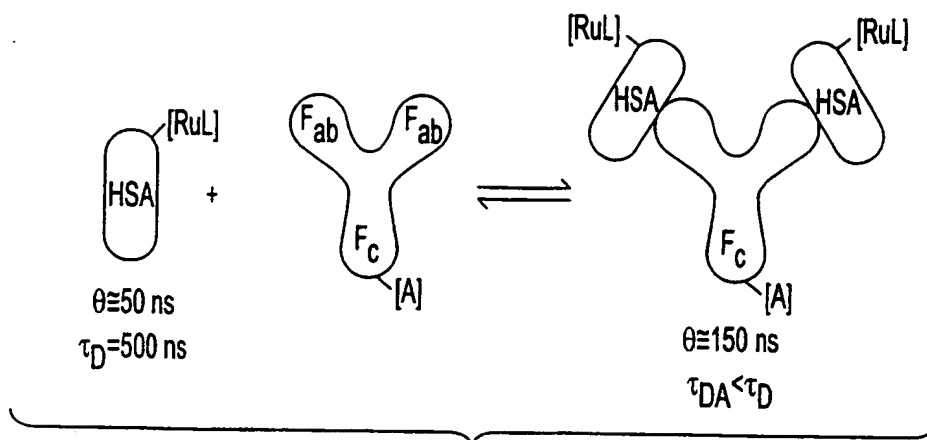


FIG. 16A

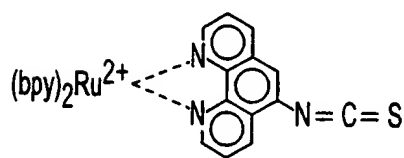


FIG. 16B

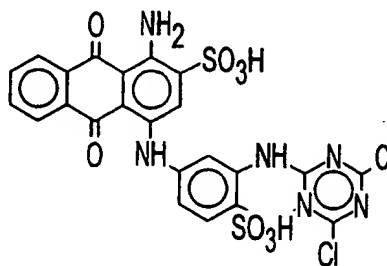


FIG. 16C

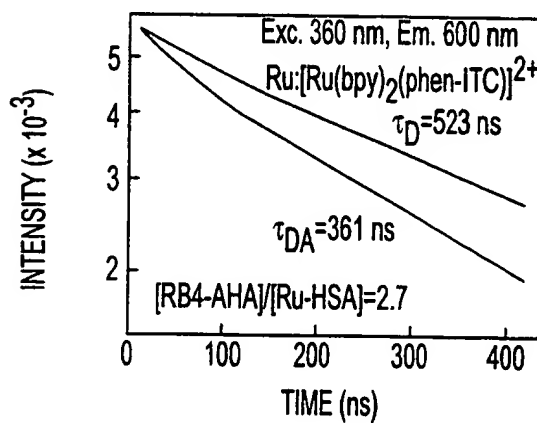


FIG. 16D

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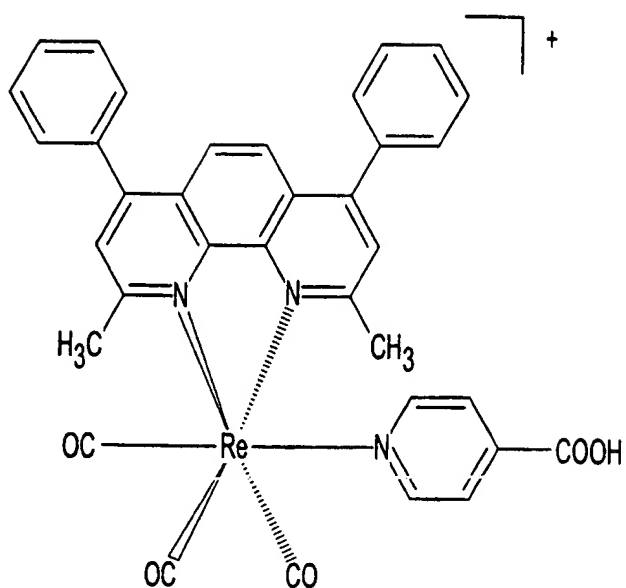


FIG. 17

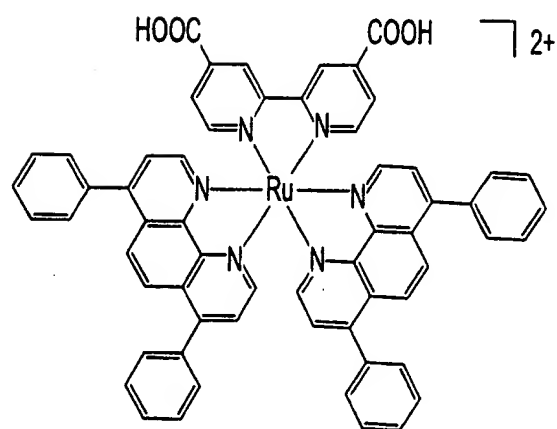


FIG. 18

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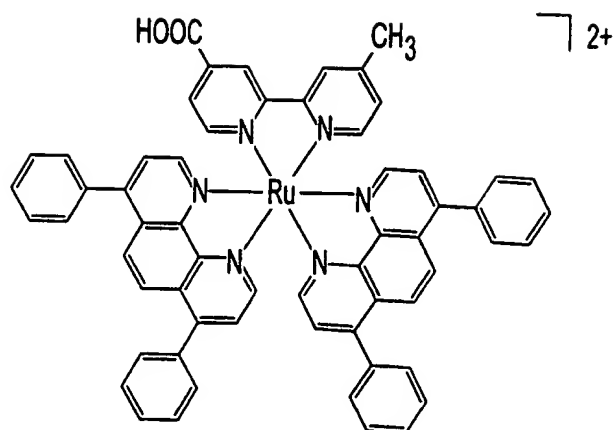


FIG. 19

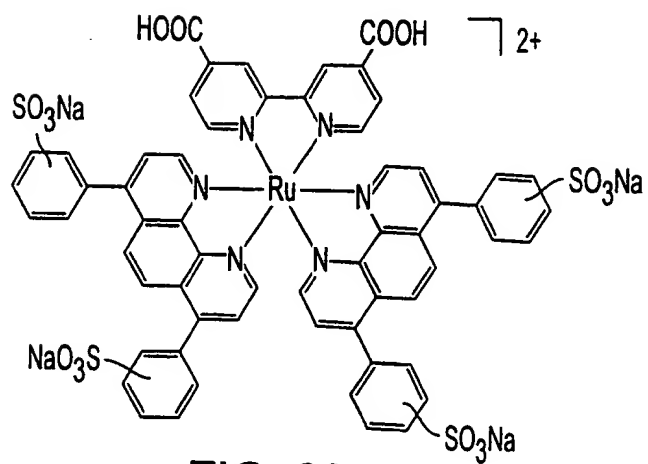


FIG. 20

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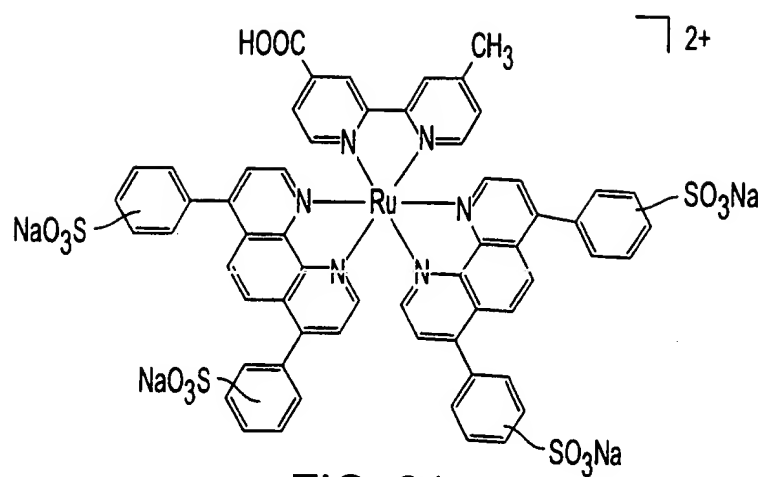


FIG. 21

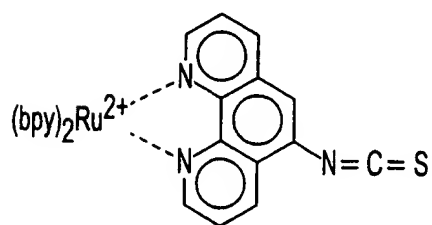


FIG. 22

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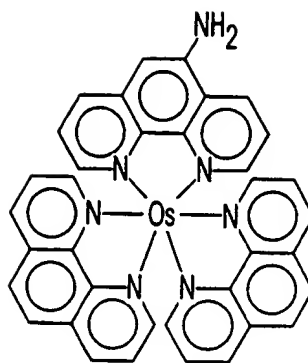


FIG. 23

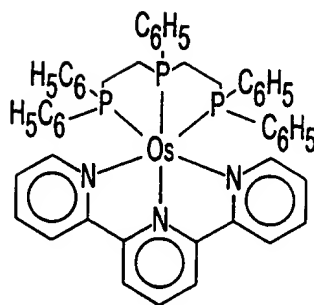


FIG. 24

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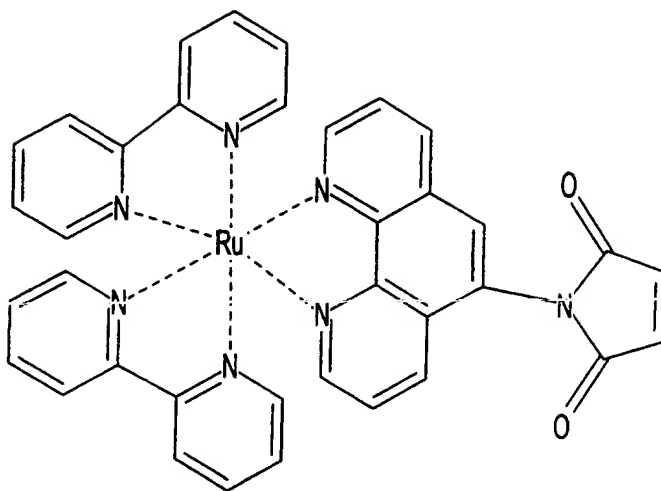


FIG. 25

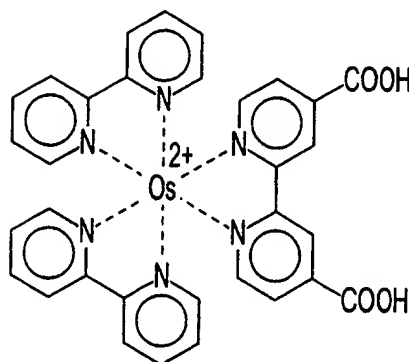


FIG. 26

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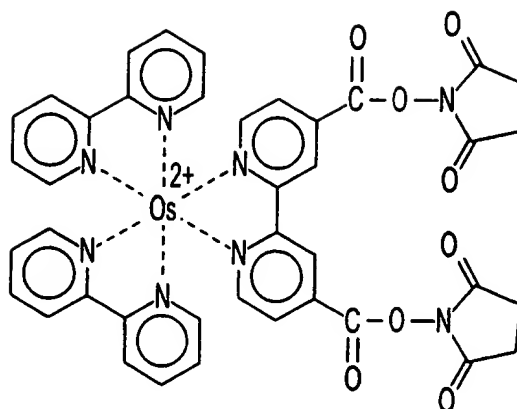


FIG. 27

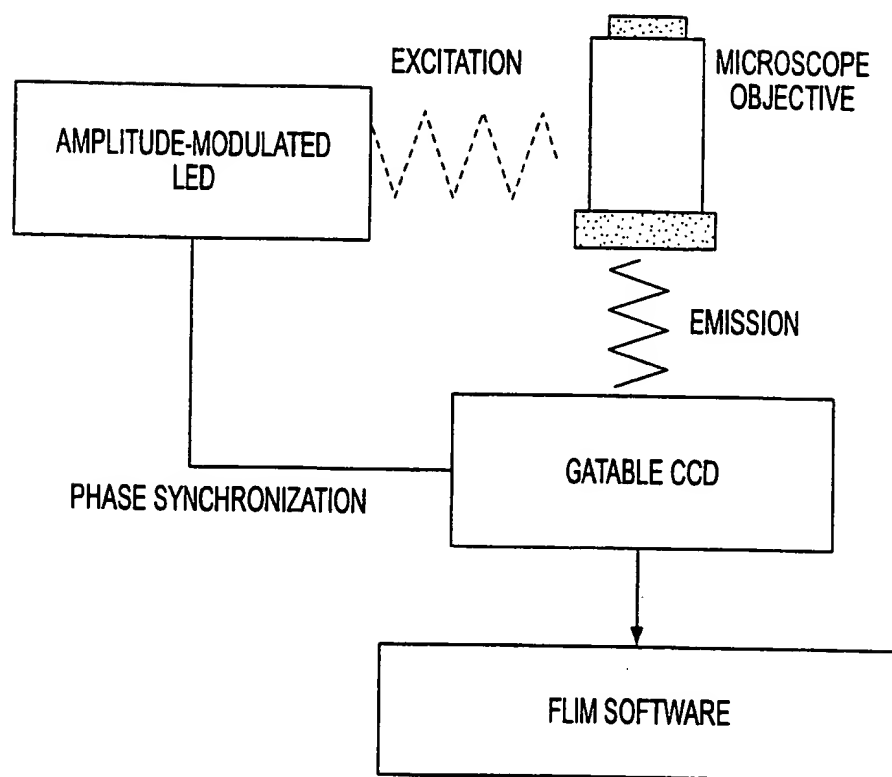


FIG. 28



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/00781

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : C12Q 1/68; C07H 21/02, 21/04; C12N 15/00

US CL : 435/6; 536/23.1, 24.3; 935/ 76, 77, 78

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 536/23.1, 24.3; 935/ 76, 77, 78

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DENUN et al. In Situ Hybridization: A Valuable Tool in Diagnostic Pathology. APMIS. 1992, Vol. 100, pages 669-681, see the entire document.	1-17, and 19-33
Y	US 5,660,991 A (LAKOWITZ et al) 26 August 1997, see the entire document.	1-17, and 19-33
Y	COLLINS et al. Identification of Sperm and Non-Sperm Male Cells in Cervicovaginal Smears using Fluorescence In Situ Hybridization: Applications in Alleged Sexual Assault Cases. Journal of Forensic Sciences. November 1994, Vol. 39, No. 6, pages 1347-1355, see the abstract.	7 and 25

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

08 MARCH 1999

Date of mailing of the international search report

24 MAR 1999

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

ETHAN WHISENANT

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/00781

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DIRKS et al. Simultaneous Detection of Different mRNA Sequences Coding for Neuropeptide Hormones by Double In Situ Hybridization Using FITC- and Biotin-labeled Oligonucleotides. The Journal of Histochemistry and Cytochemistry. 1990, Vol. 38, No. 4, pages 467-473, see the abstract.	8 and 26
Y	ZHANG et al. Genetic Typing by Capillary Electrophoresis with the Allelic Ladder as an Absolute Standard. Analytical Chemistry. 01 September 1996, Vol. 68, No. 17, pages 2927-2931, see the entire document.	16
Y	VOSKOVA-GOLDMAN et al. DMD-Specific FISH Probes are Diagnostically Useful in the Detection of Female Carriers of DMD Gene Deletions. Neurology. June 1997, Vol. 48, pages 1633-1638, see the abstract.	9 and 27
Y	The Stratgene Catalog. Gene Characterization Kits. 1988, page 39.	33
Y	MALAK et al. Long-Lifetime Metal-Ligand Complexes as Luminescent Probes for DNA. Journal of Fluorescence. February 1997, Vol. 7, No. 2, pages 107-112, see the entire document.	19

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/00781

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

USPATFULL, EUROPATFULL, JAPIO, WPIDS, CAPLUS, MEDLINE

search terms: Fluoresce? and (probe? or primer?) and metal

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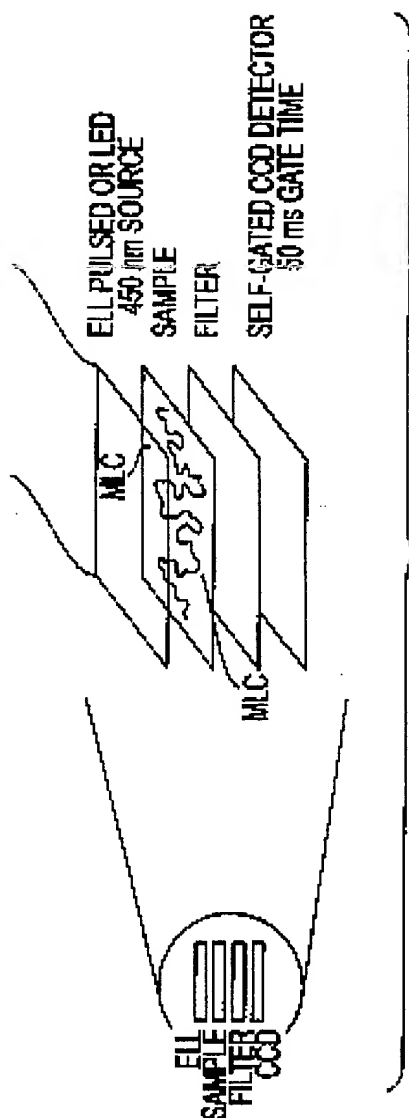


FIG. 1

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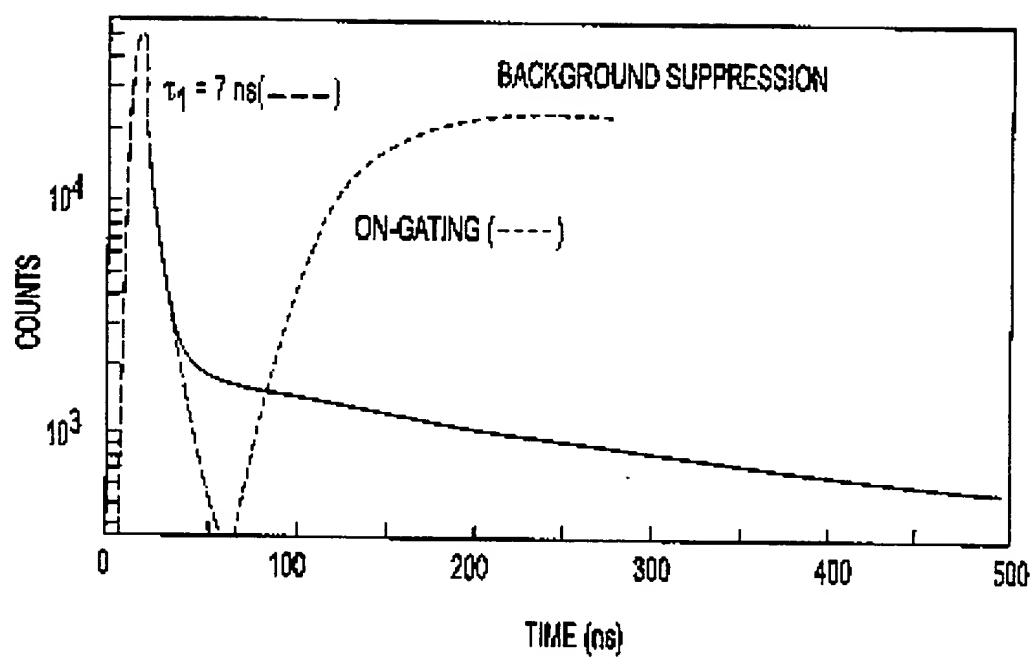


FIG. 2

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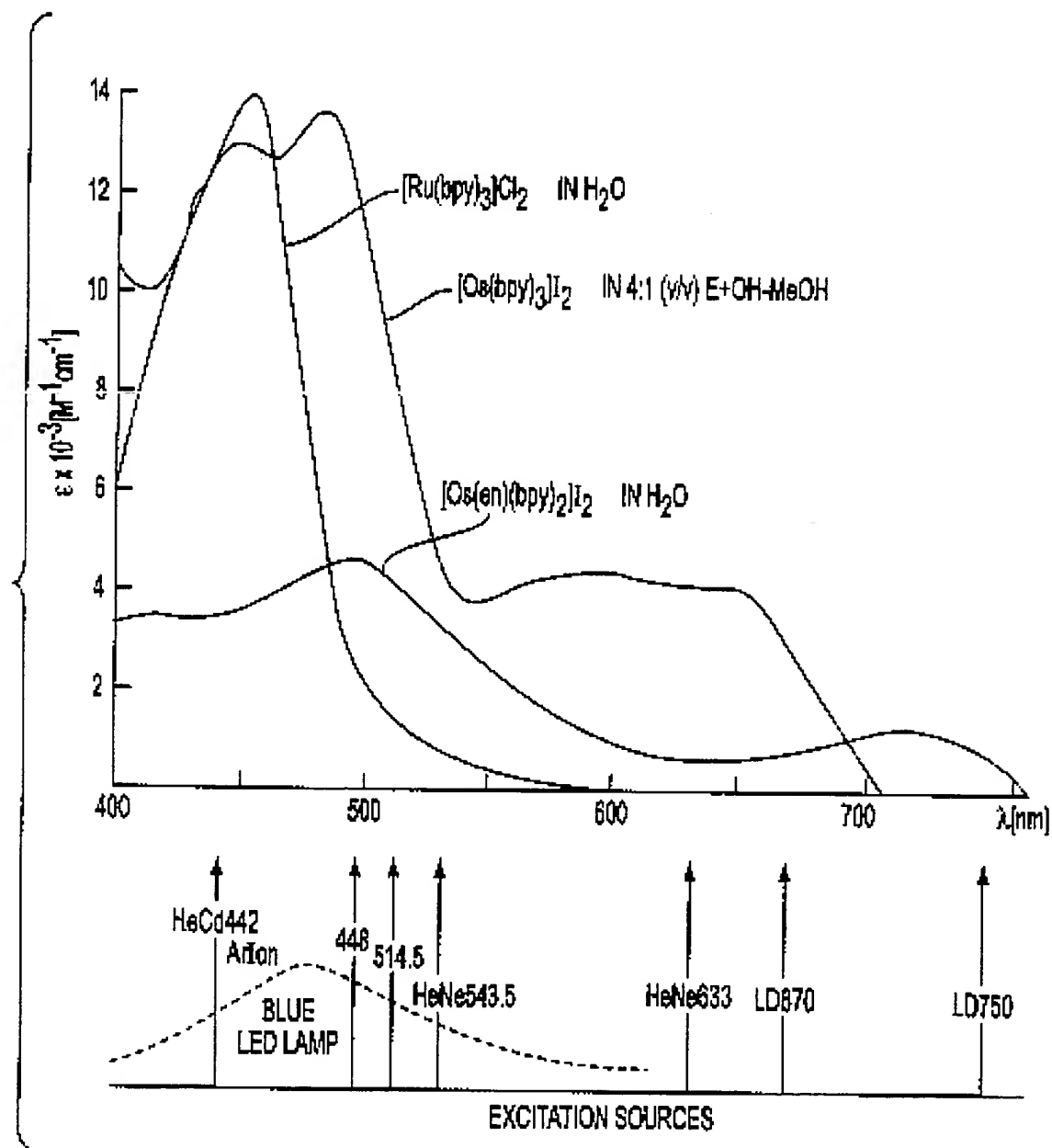


FIG. 3

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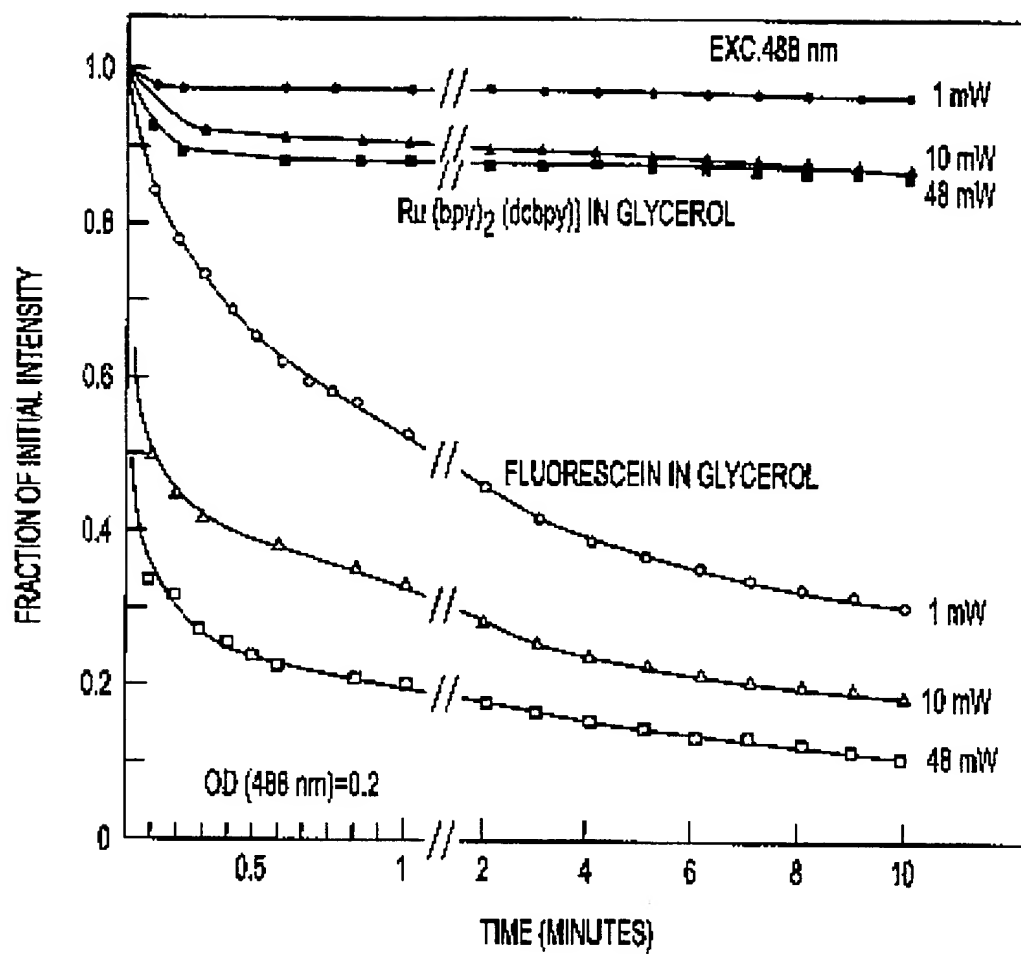


FIG. 4



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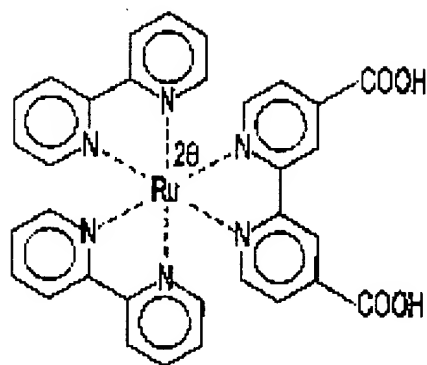


FIG. 5A

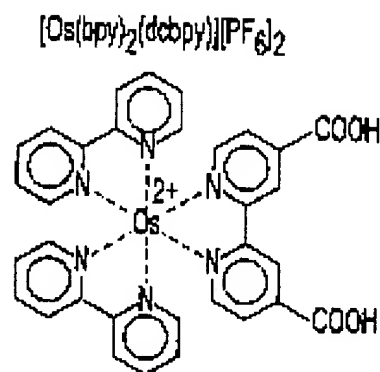


FIG. 5B

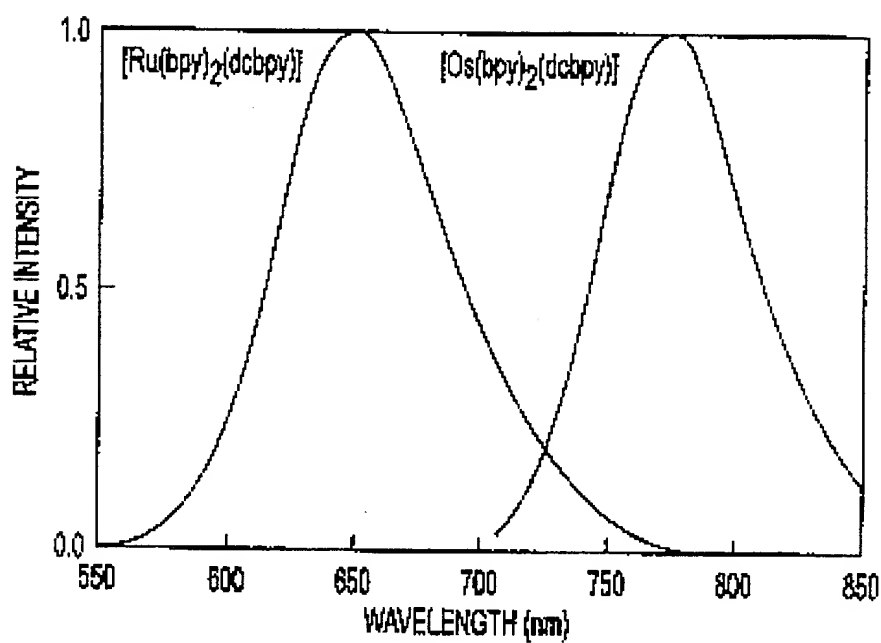


FIG. 5C

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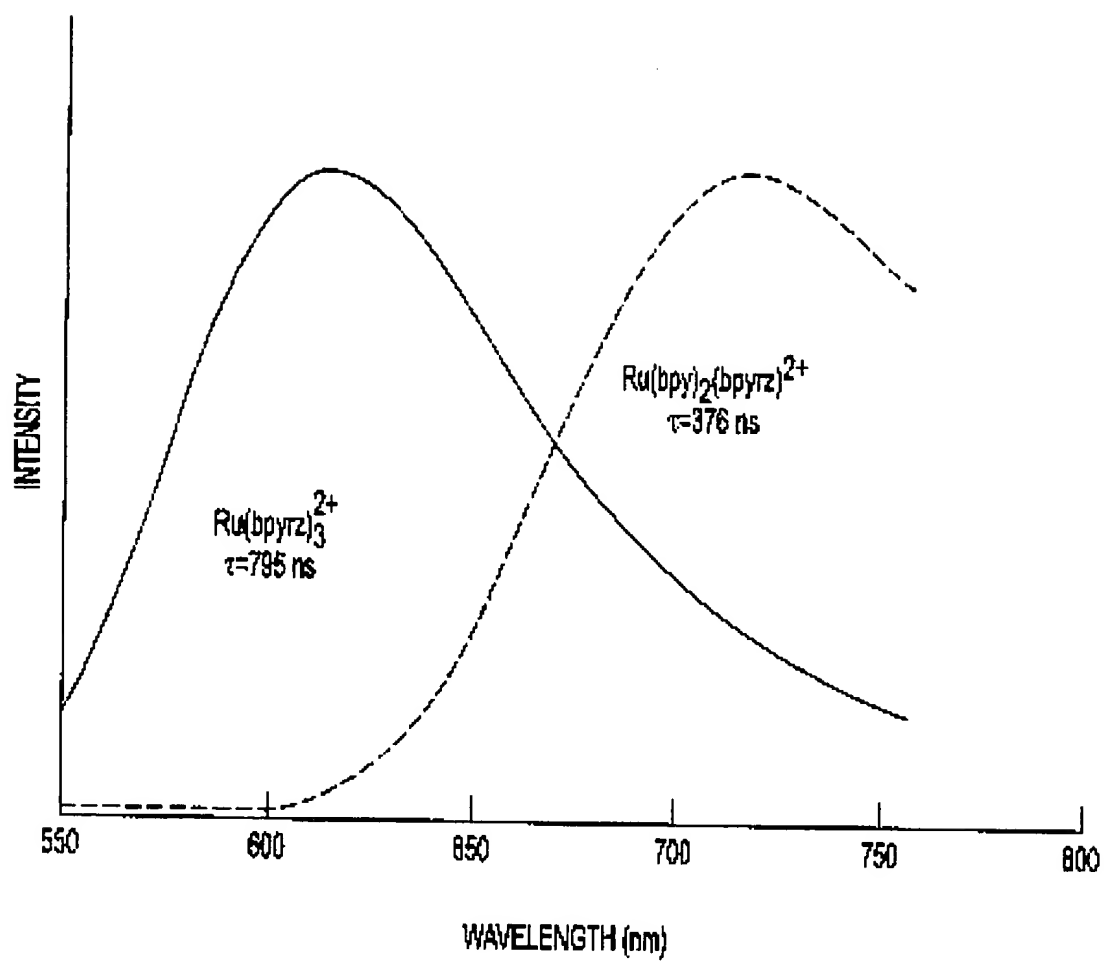


FIG. 6

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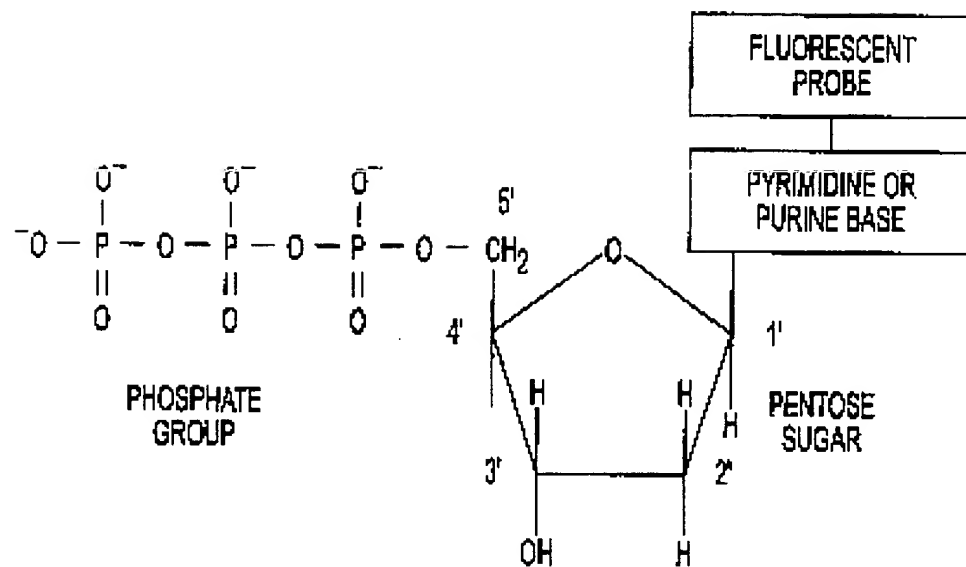


FIG. 7

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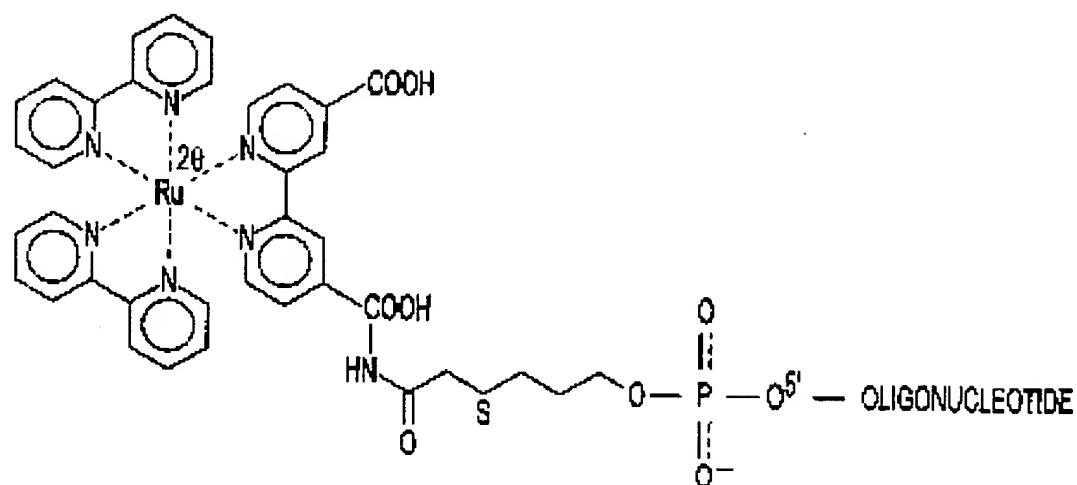


FIG. 8A

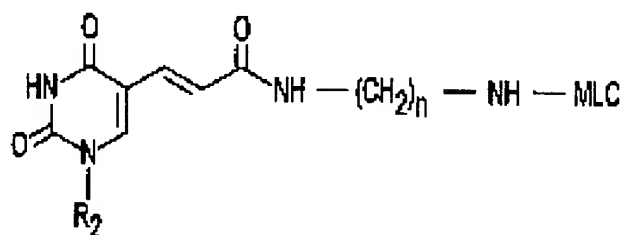


FIG. 8B

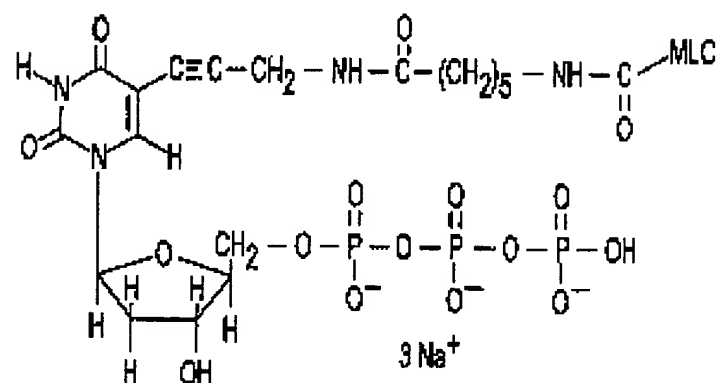


FIG. 8C

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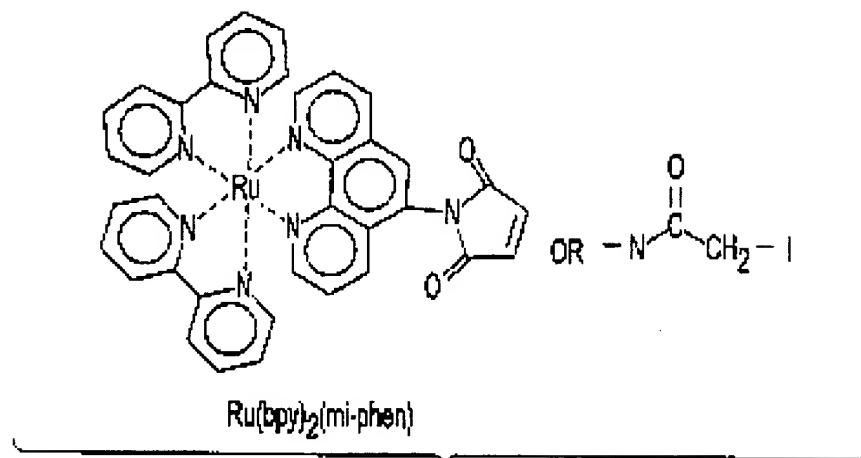


FIG. 9A

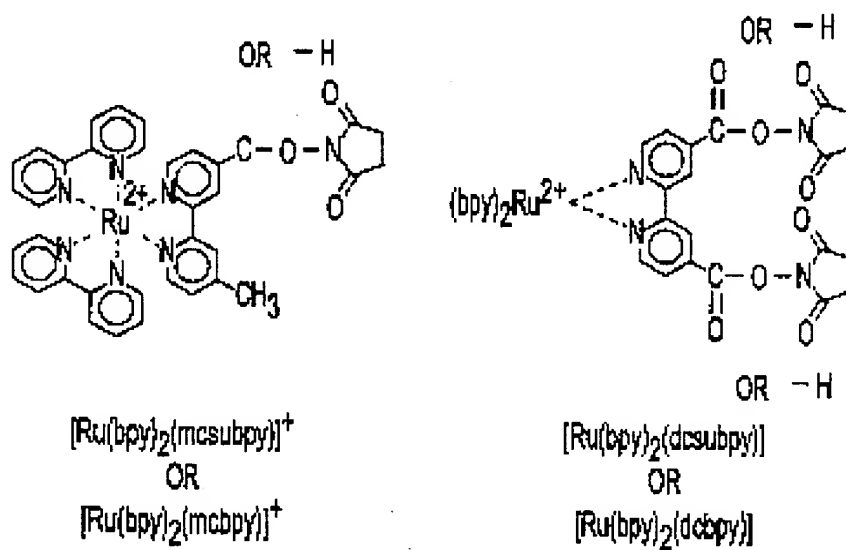


FIG. 9B

FIG. 9C

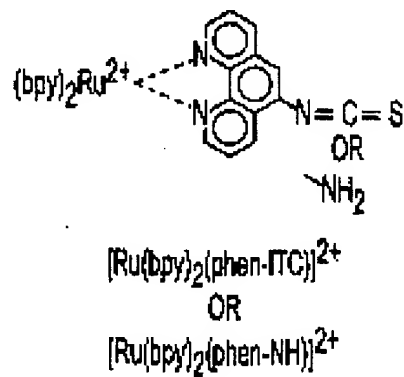


FIG. 9D

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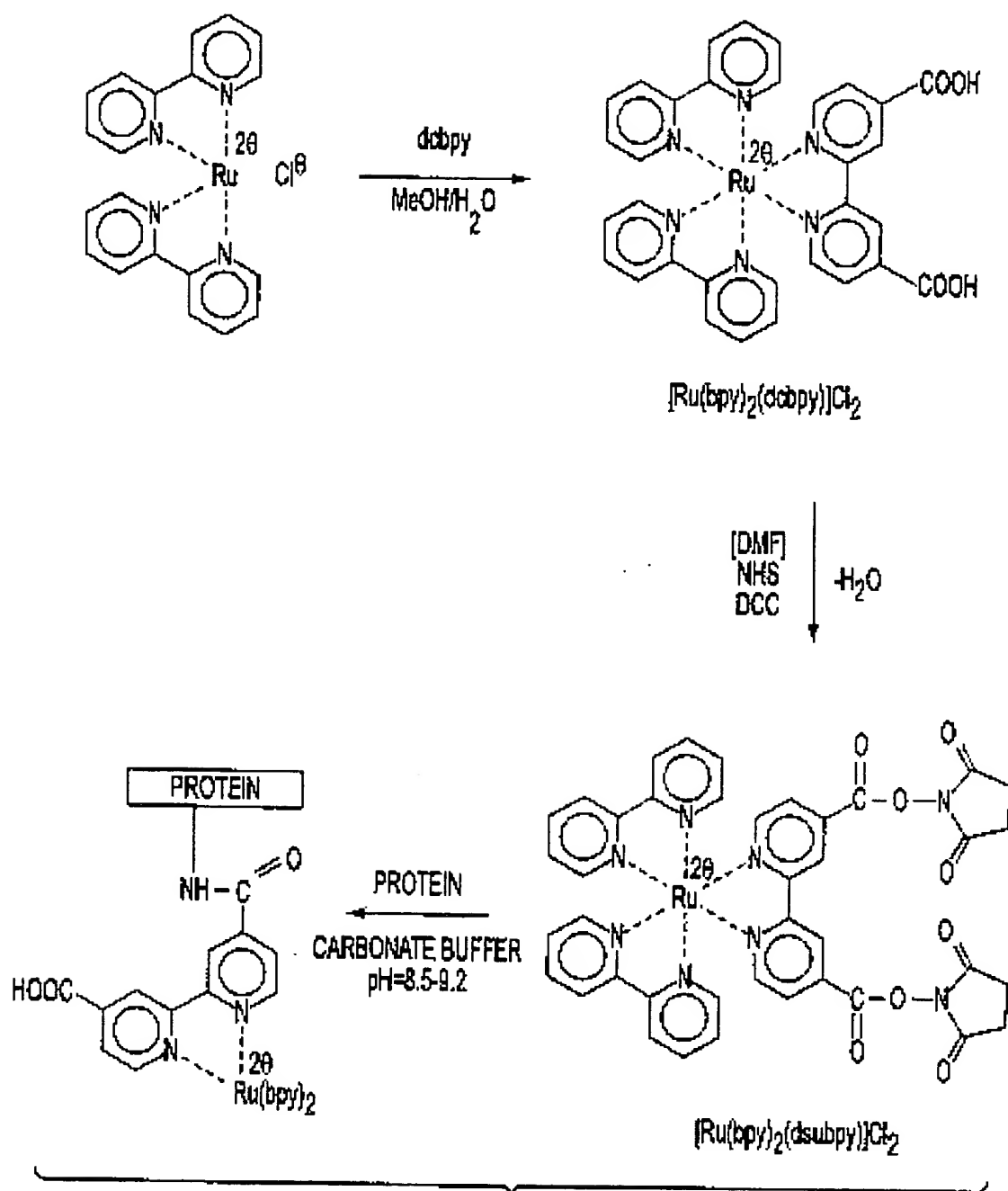


FIG. 10

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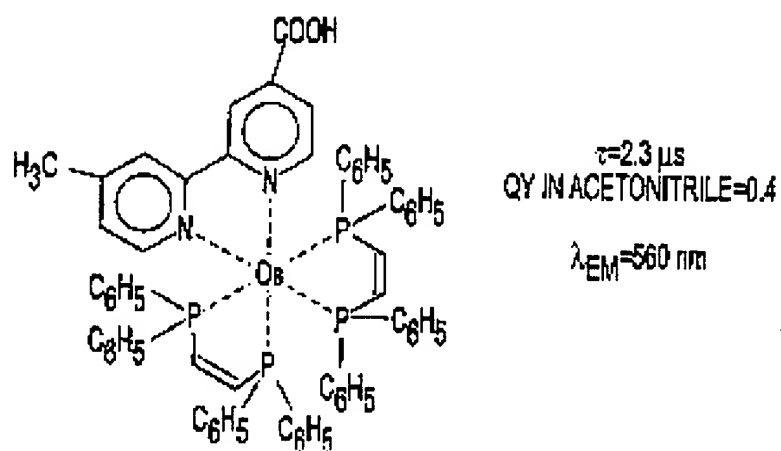


FIG. 11A

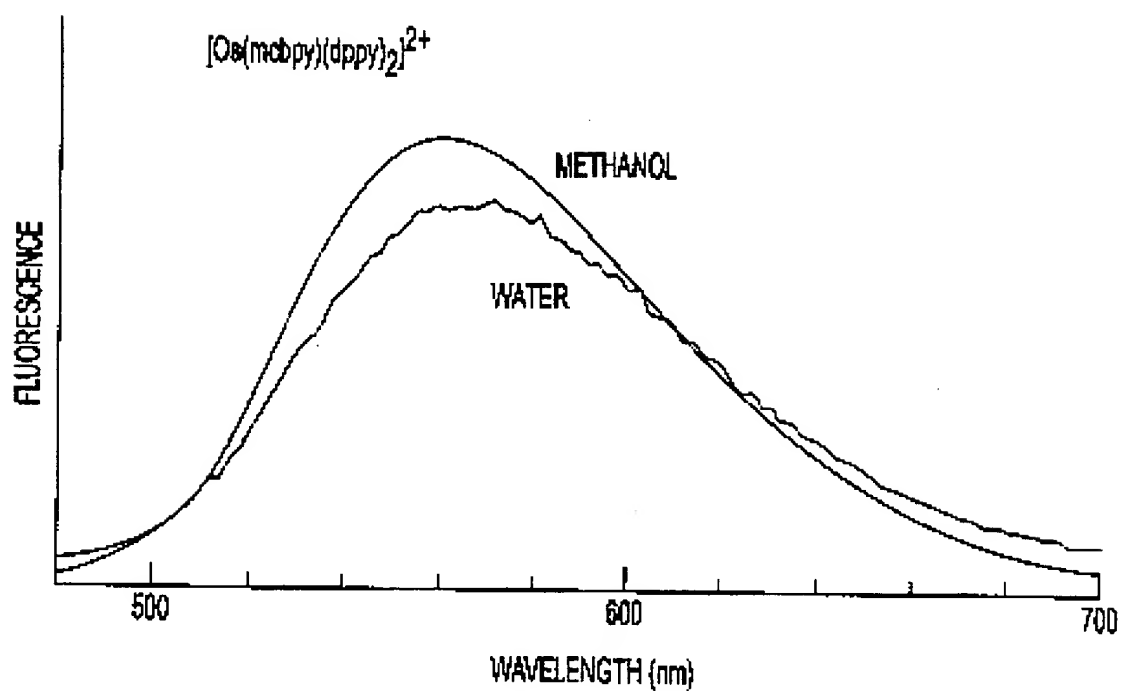


FIG. 11B

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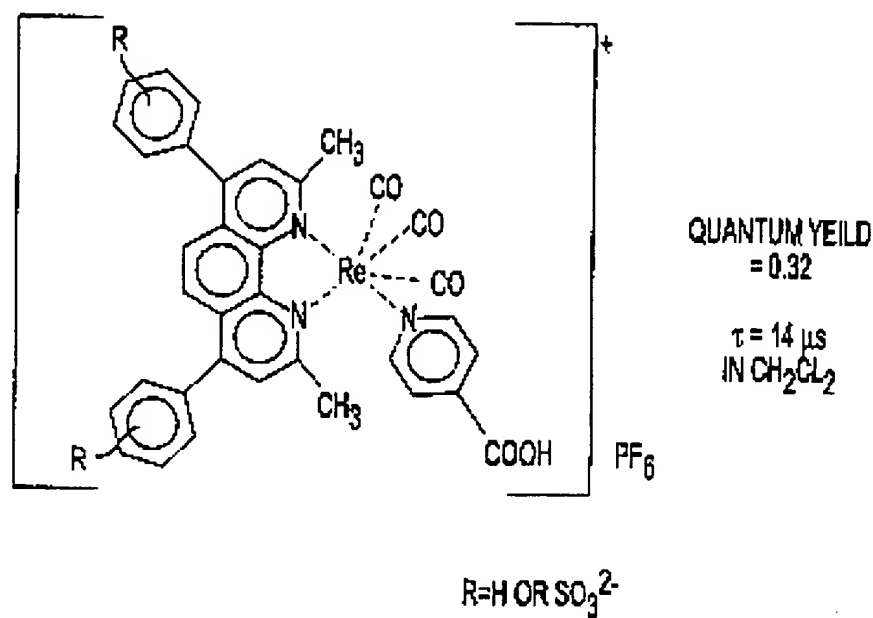


FIG. 12A

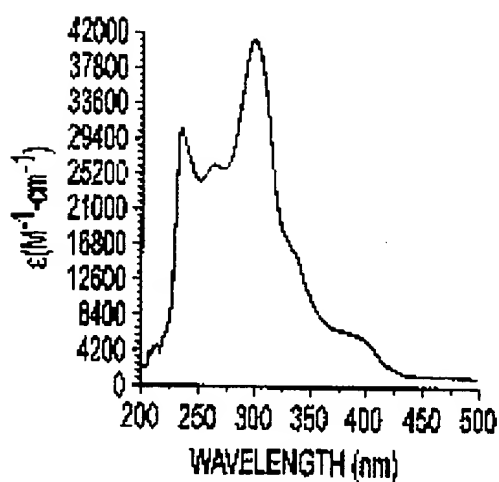


FIG. 12B

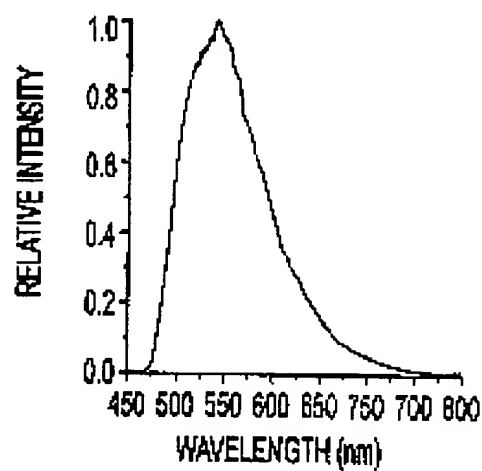


FIG. 12C



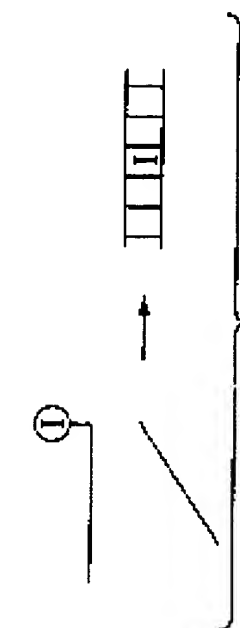


FIG. 13D

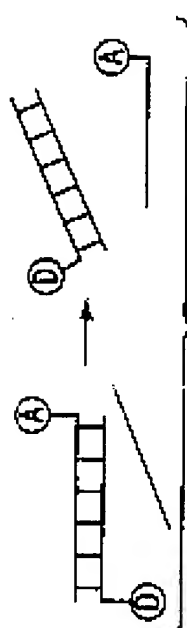


FIG. 13E

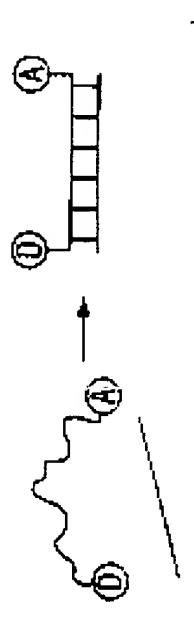


FIG. 13F

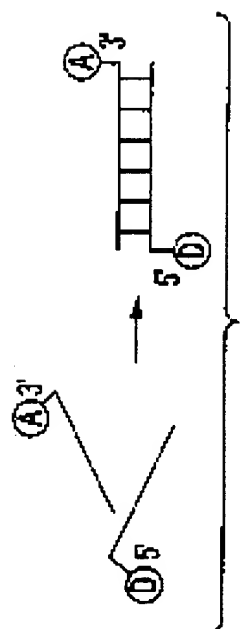


FIG. 13A

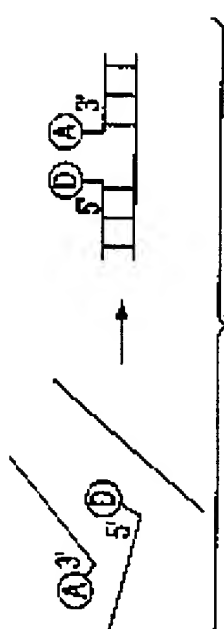


FIG. 13B

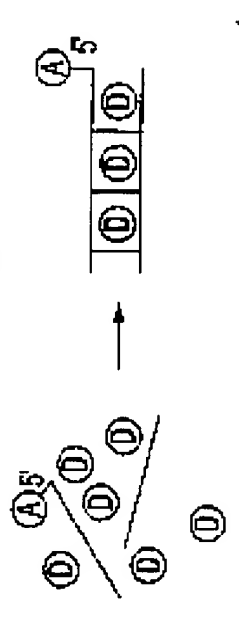


FIG. 13C

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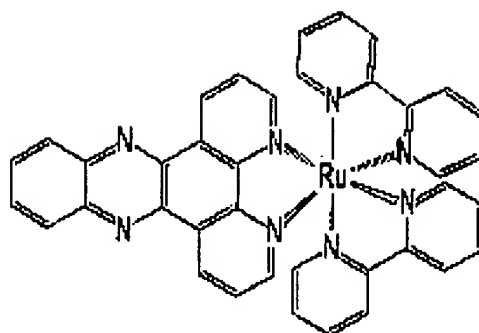


FIG. 14A

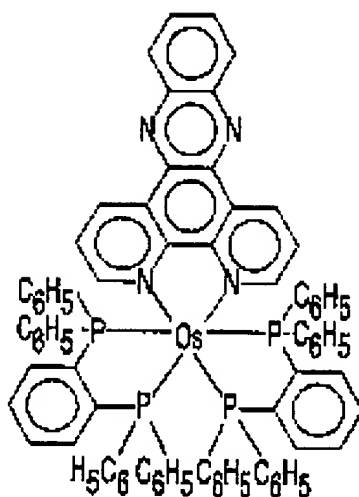


FIG. 14B

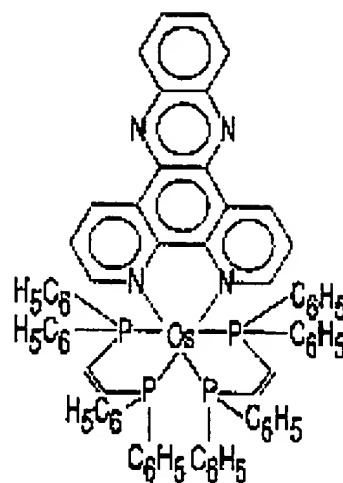
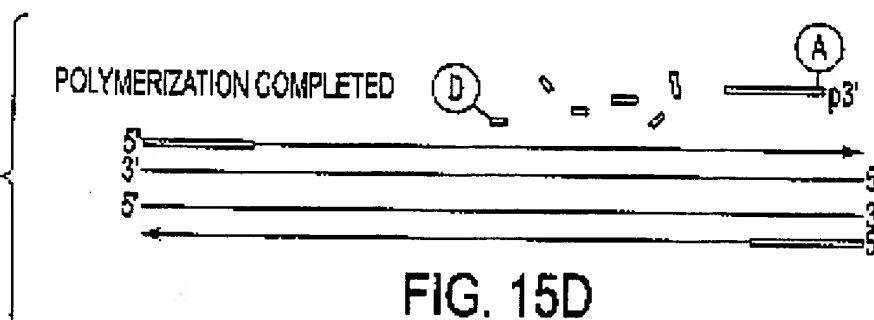
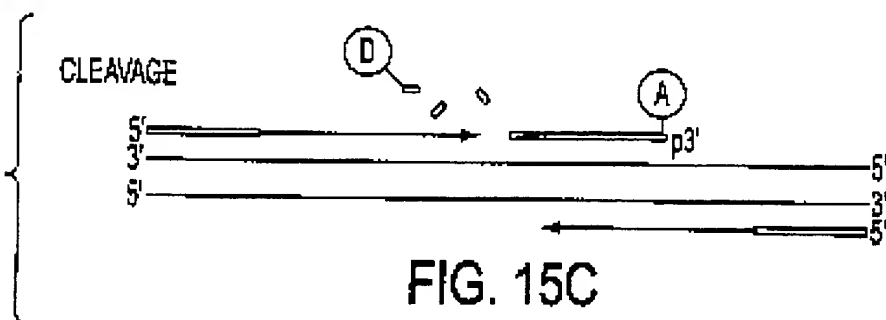
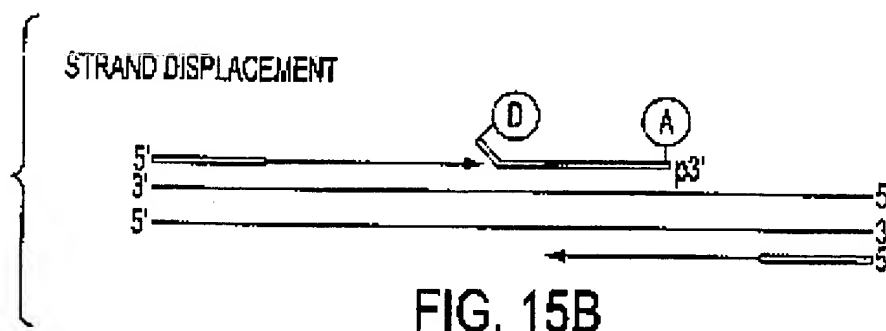
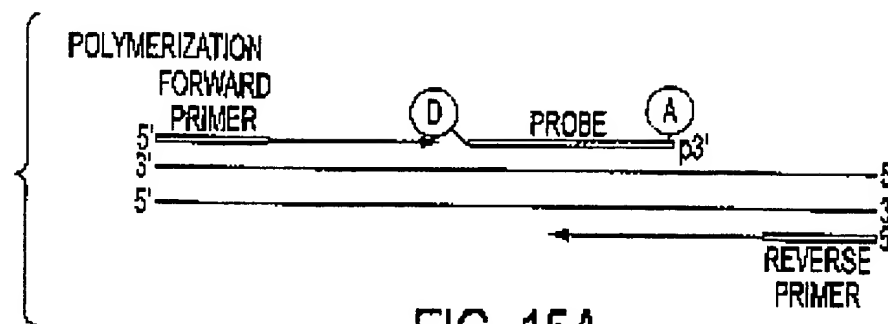


FIG. 14C

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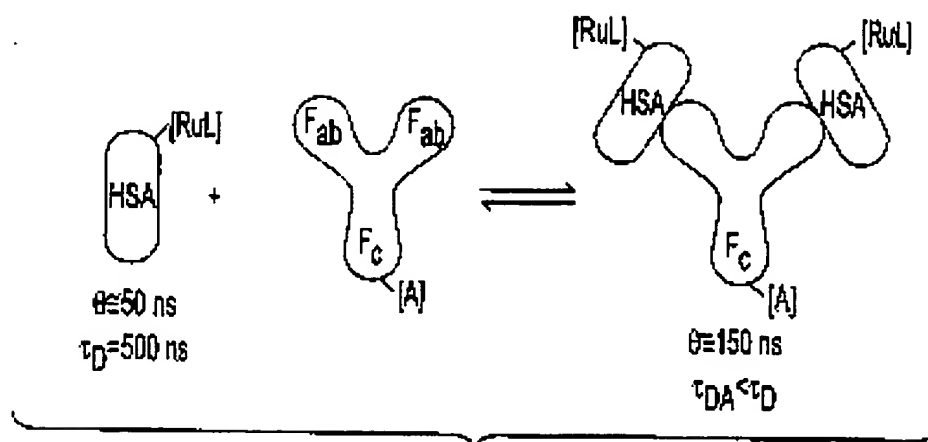


FIG. 16A

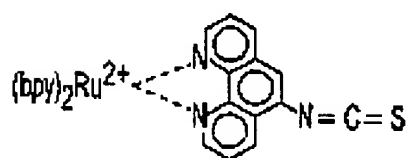


FIG. 16B

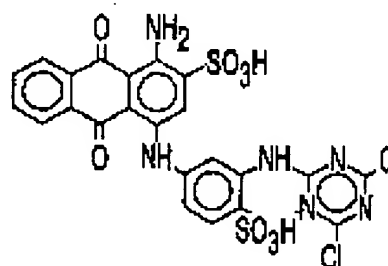


FIG. 16C

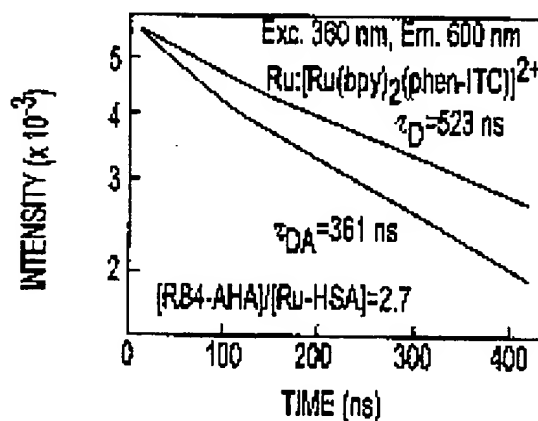


FIG. 16D

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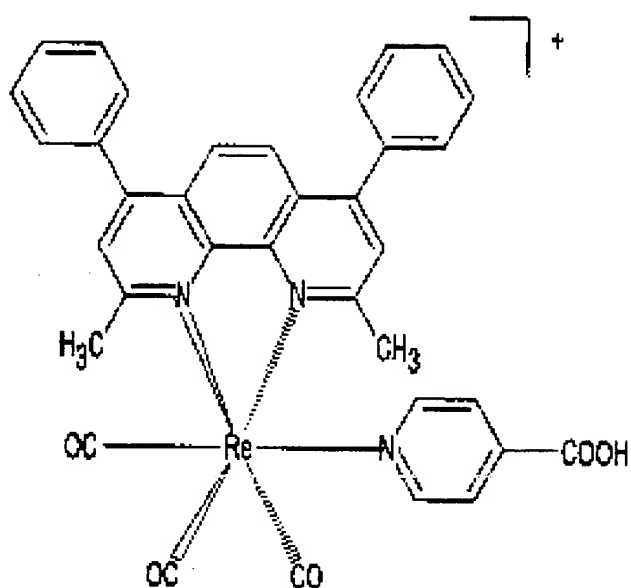


FIG. 17

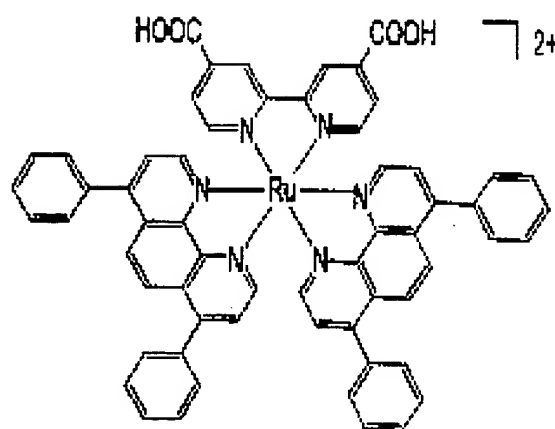


FIG. 18

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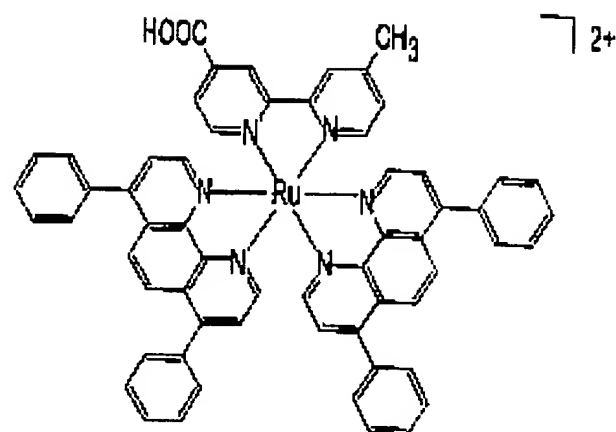


FIG. 19

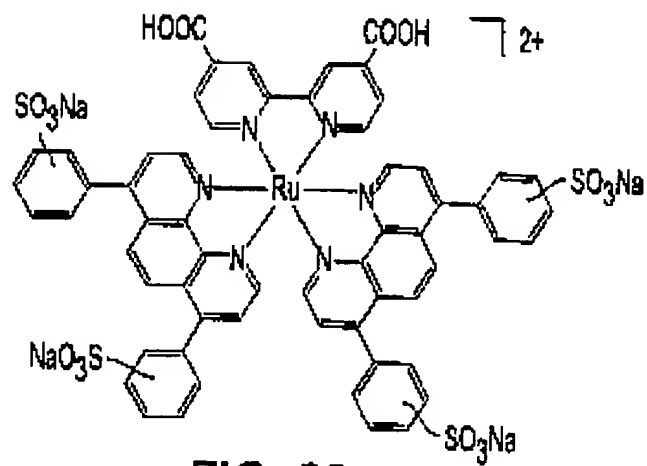


FIG. 20

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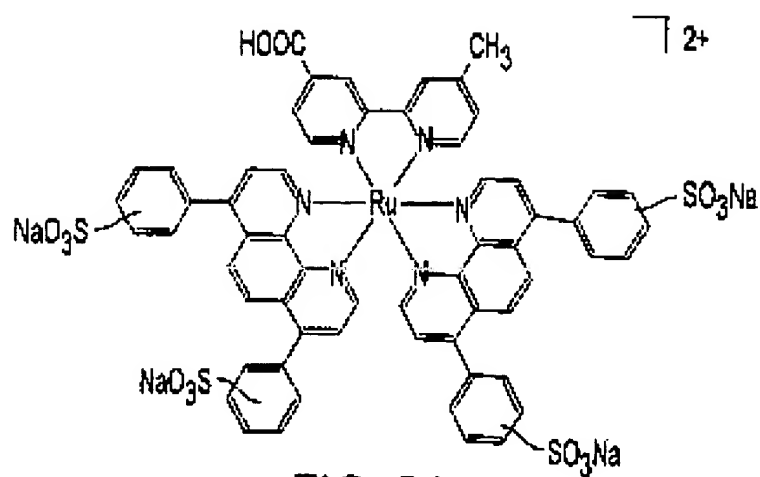


FIG. 21

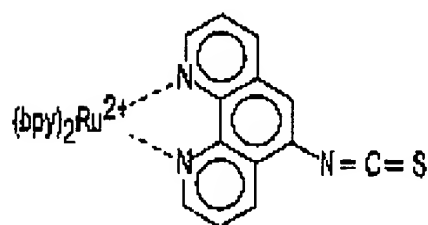


FIG. 22

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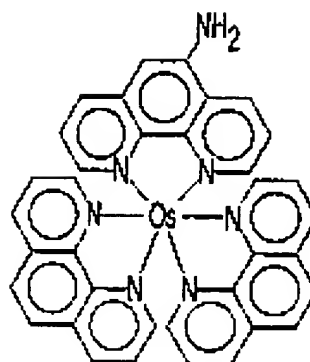


FIG. 23

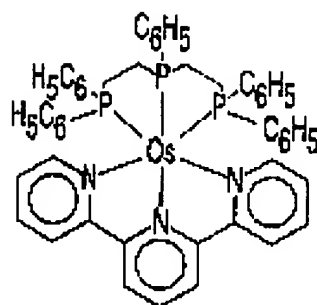


FIG. 24



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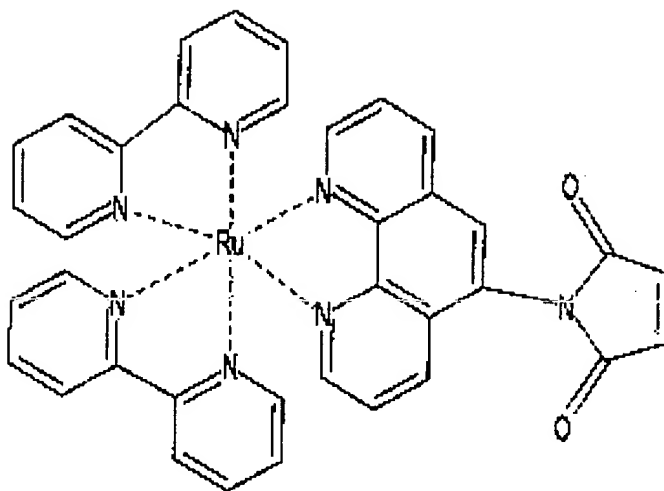


FIG. 25

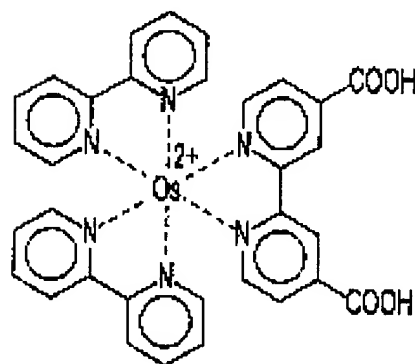


FIG. 26

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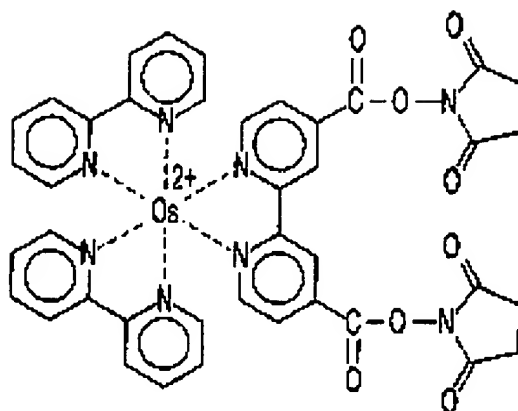


FIG. 27

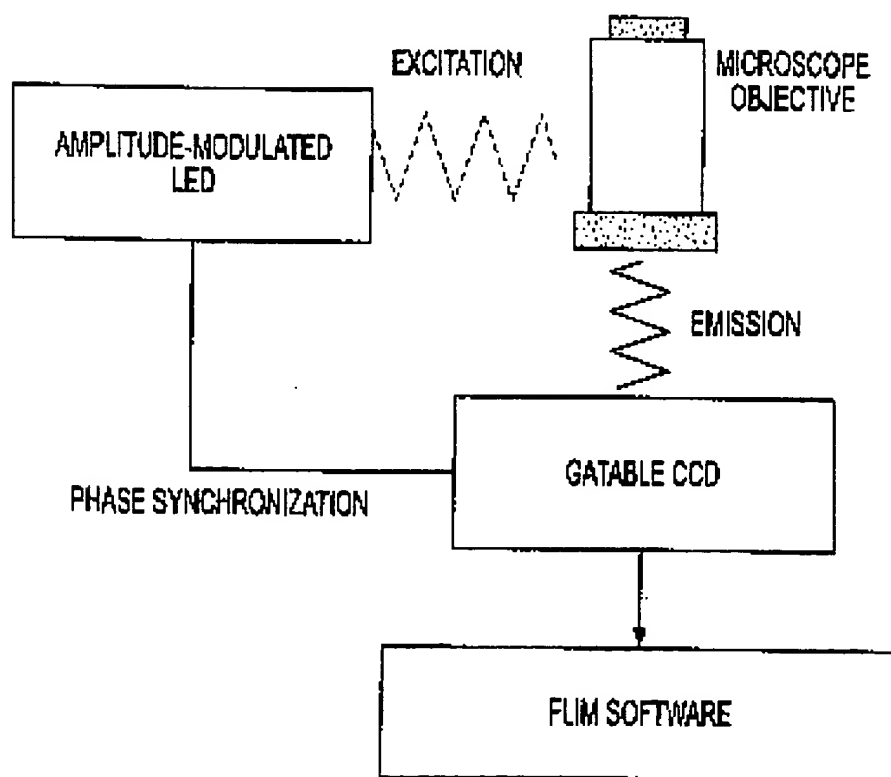


FIG. 28